2016

# A PRELIMINARY LONGITUDINAL STUDY OF VOCAL LEARNING IN LATE TUTORED JUVENILE ZEBRA FINCHES USING FUNCTIONAL MAGNETIC RESONANCE IMAGING

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Submitted in Partial Fulfillment of the Prerequisite for Honors in Neuroscience

May 2016

# Acknowledgements

It takes a village to raise a thesis so I want to thank everyone who has ever helped me (and this project) get to this point.

I first and foremost want to thank my amazing advisors, **Sharon Gobes** and **Nancy Kolodny**. It has been a tremendous privilege to have them as my mentors and I could not have asked for better ones. My life was forever changed when I was asked to be part of this project my sophomore year. The independence and trust they gave me over the years, allowing me to carry the project forward, accepting and trusting my growing expertise, have not gone unnoticed. If not for their endless support and guidance, I would have quit long ago when the project seemed impossible. They have pushed and supported me through the last three years of my life and during times that felt truly bleak. I have learned so much. I appreciate all you've done for me, and the care you have had for me in my research, academic and personal endeavors.

To **Sharon**, I can't thank you enough for giving me this opportunity, for being my mentor, for guiding me towards becoming a scientist. I truly would not be the person I am without you. It has not always been an easy journey, but it has been a worthwhile one. I will carry forward the lessons you have taught me in both the lab and the classroom.

To **Nancy**, it has been such a pleasure and honor to be in your lab. I have learned so much from you and your expertise and passion have fueled my budding interest in imaging. It will be hard to forget your smile at lab meetings and endless positivity and cheer, but they will not be hard to miss.

I would also like to thank my other thesis committee members: Professors **Margaret Keane, Barbara Beltz** and my honors visitor, **Megan Nunez**. Thank you for your continued support in this thesis. Thank you for being there for me through tough schedules and tougher problems.

Additionally, this project and thesis would not have been possible without the additional help from **George Dai, Elaine Igo, John DeBolt and Larry Knowles**. Thank you for being 'oncall' when the magnet doesn't work, speaker wires snap and plastic beds crack. The success of the project would be naught without you all. I'd also like to extend a special thank you to **Val LePage** and **Pat Carey** for their excellent help in the care of our zebra finches.

Thank you to my parents, **Jenny Petkova** and **Pavel Petkov**, for the endless support and for always listening to me explain and complain about what I do. Thank you for your advice, your consoling words and encouragement. Thank you for always encouraging and helping me pursue my dreams by every means possible, even when I am not sure what they are. Thank you for teaching, educating, and inspiring me.

I'd also like to thank all my wonderful friends. To my alphas, (in no particular order) **Giovi Mier, Sam Goldstein, Cayla Fromm, Madeline Gorchels, Emma Howey, Dulce Gonzalez, Rita Mary Hennigan**, thank you for your unconditional love, for being my sisters and for these four amazing years. To my fellow Sage Sufferers, **Amy Yuan** and **Maggie Feng** for all the nights of laughs and work in Sage Lounge. To my neuro triad members **Blair Daniel** and **Isabelle Rosenthal**, thank you for geeking out about neuroscience with me the last three years and always helping me find the bright side of research. To my lab mates, **Rie Maeda** and **Alexa Pagliaro**, I thank you for one of the best summers of my life. To current and previous lab members of both the Gobes and Kolodny labs, I thank you for your support, advice, and endless stimulating conversations. I want to extend a special thank you to **Sarah Zemlok** and **Palig Mouradian** who continuously encouraged me when I took over the project and was unsure of myself.

Finally, I'd like to thank **Arthur Riegel and the Riegal lab** at the Medical University of South Carolina for culturing my interest in research. They took in a nervous first year and gave me one of the most life changing summers. I always intended on keeping my promise in thanking you in my first thesis.

# Table of Contents

# Abstract

Language is often considered what defines us as human, yet there is not a clear understanding of how language develops in young children. Zebra finches (*Taeniopygia guttata)* serve as a suitable animal model to study neural mechanisms underlying model vocalizations that are similar to human speech. Due to developmental and neuroanatomical similarities between zebra finches and humans, zebra finches prove to be an excellent model. Male zebra finches, like humans, acquire acoustically complex vocalizations through auditory experience and practice during development. In zebra finches, song acquisition is commonly investigated using immunocytochemical and electrophysiological methods, but recently functional magnetic resonance imaging (fMRI) in zebra finch research has also gained traction. While much is understood regarding where song memories might be stored and how the brain changes in response to song learning, fMRI uniquely allows for a noninvasive and longitudinal approach to investigating specific changes in the zebra finch brain during the song learning process. In this study, we observe longitudinal changes in neural responses to tutor and conspecific song before and after tutoring during the sensorimotor period. Using region of interest (ROI) analysis, we found that tutor song during adulthood elicited significant activation compared to silence in auditory regions MLd and NCM. Additionally, we found a correlation between neural activation in both left and right NCM during adulthood in response to tutor song and similarity to the tutor song during adulthood. Finally, we found a correlation between activation in left MLd the first time a juvenile heard tutor song and its similarity to the tutor song at adulthood, suggesting a predictive relationship between response to song during development and learning outcomes in adulthood.

# Introduction

***Zebra finches as models for vocal learning and memory:***

Male zebra finches acquire species-typical vocalizations through a highly social, trial-and-error learning process, similar to how human children acquire speech (Goldstein et al., 2003; Kuhl, 2003). Zebra finch song is considered analogous to human speech because songbird vocalizations share many qualities with human speech, such as acoustic complexity and social and behavioral saliency (Doupe and Kuhl, 1999). Zebra finch songs are made of multisyllabic 'motifs' that are often repeated during a song. Motifs have acoustic complexity as each 'syllable' has a specific pitch, frequency and temporal identity (Doupe and Kuhl, 1999) (Figure 1). Human speech is a social communication tool and song is similarly socially salient with a specific function in communication: male song is used as a mating signal and to secure territory (Doupe and Kuhl, 1999). Young zebra finches have an innate preference for conspecific sounds, but learning and production of species-typical song is a complex process that is highly dependent on auditory input. Exposure to song from an adult male during development is necessary for juvenile zebra finches to develop the temporal sequence and acoustic properties that resemble adult vocalizations (Doupe and Kuhl, 1999). Without auditory experience with a 'model' song, male zebra finches are unable to learn and produce a stable, species-typical song and instead retain undeveloped vocalizations (Bolhuis et al., 2010; Doupe and Kuhl, 1999). Similarly, humans require social experience to acquire and use language (Doupe and Kuhl, 1999).

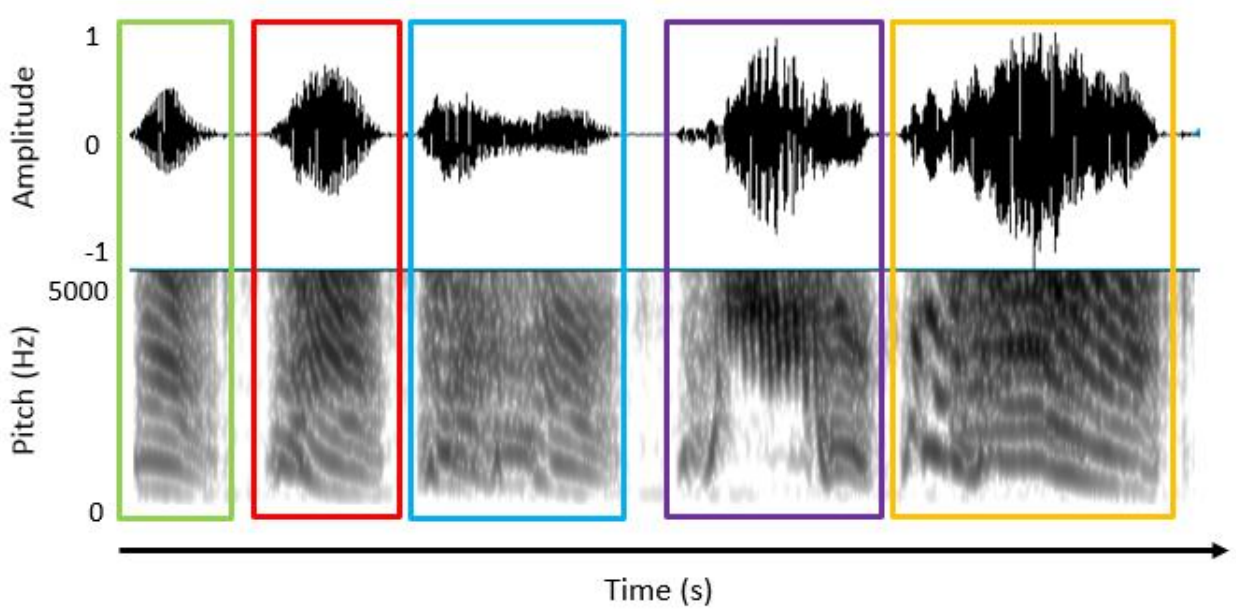


**Figure 1: Representative motif of adult, crystallized song.** Top shows sound signal amplitude as a function of time. Bottom shows the spectrogram, the sound signal pitch as a function of time. Colored boxes show the different and distinct syllables with characteristic pitch, frequency and length.

***Developmental Similarities:***

Zebra finches and humans share a similar sequence of events in vocal acquisition (Bolhuis and Gahr, 2006; Bolhuis et al., 2010; Doupe and Kuhl, 1999; Mooney, 2009; Woolley, 2012). Early in development, humans and zebra finches listen to and memorize adult vocalizations; this is a period of perceptual learning (Figure 2). In juvenile zebra finches, this period is defined by high receptivity to song, but birds are not usually beginning to practice singing songs themselves (Woolley, 2012). It has been shown that if juvenile birds are exposed to tutor song later in the perceptual learning phase, their adult song will be more similar to the tutor song (Roper and Zann, 2006). Human babies experience a comparable period in development during which they listen to speech without producing any vocalizations. During the subsequent sensorimotor learning phase, both zebra finches and humans begin to develop their own vocalizations through practice (Figure 2). When human infants begin to coo, they produce short monosyllabic sounds (ma ma, da da, etc).

Subsong, the songbird equivalent to human cooing, consists of a similar pattern of repeating single syllables (Bolhuis et al., 2010). Human infants continue on by practicing babbling and adding complexity by stringing sounds to make words. Juvenile zebra finches have a similar phase of vocal experimentation, known as plastic song. In the final stage, children begin stringing words together until they are able to make fluid sentences. Likewise, through practice and continuous vocal experimentation, the juvenile male zebra finch imitates the target tutor song until it becomes a crystallized, stable song which it will sing for the rest of its life (Figure 2).

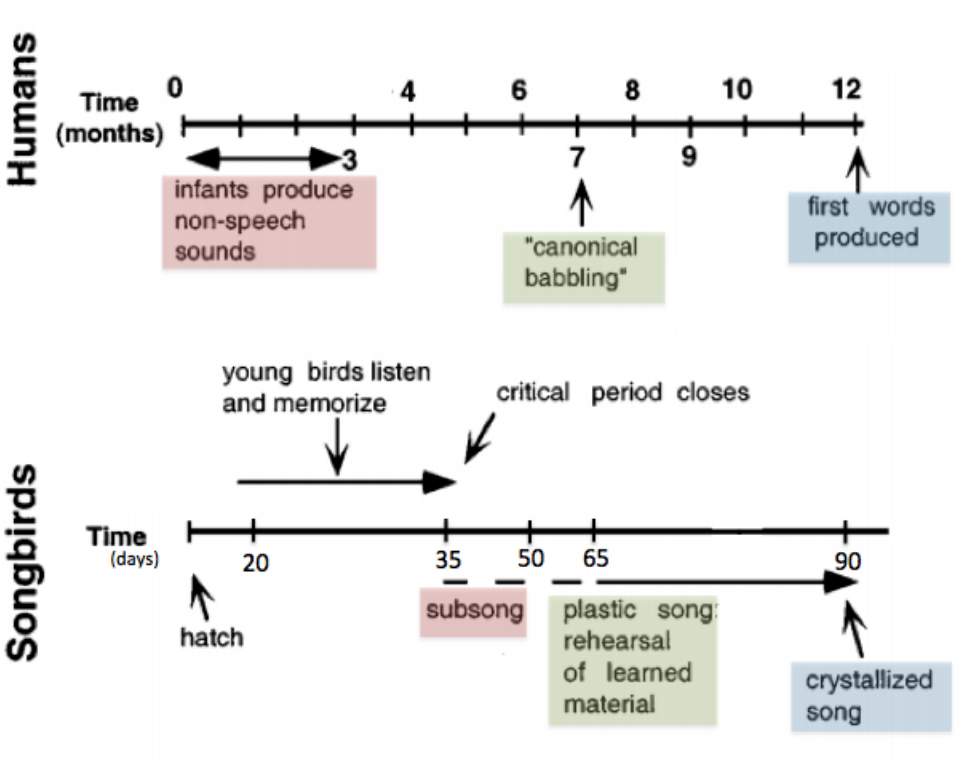


**Figure 2: Parallels between human speech and zebra finch song development**. Adapted from Doupe and Kuhl 1999.

Previous evidence suggests that during the sensorimotor learning period, zebra finches imitate a tutor song through auditory feedback and comparison of their song to a tutor song template. After a single exposure to tutor song during the sensorimotor period, juvenile zebra

finches begin to produce more structured, adult-like, vocalizations immediately after the auditory exposure. With continued exposure, vocal experimentation increases exponentially and song features such as pitch, frequency modulation, spectral continuity of frequency contours, and Weiner entropy, a measure of tonality, immediately begin to change to better imitate the memorized tutor song (Tchernichovski et al., 2001). Extensive trial-and-error vocal experimentation occurs as the tutee corrects his own song to match the tutor song. During this period of vocal experimentation, both humans and zebra finches learn more about sound 'semantics', words or song syllables, syntax, correct order and timing of words and song syllables.

Humans and zebra finches have similar 'sensitive' or 'critical' periods wherein the propensity of song or sound learning is greatest earlier in development (Bolhuis et al., 2010; Doupe and Kuhl, 1999). A zebra finch's sensitive period for song learning begins around 25-30 days post hatch (dph) (Braaten, 2010; Roper and Zann, 2006). Furthermore, zebra finches produce most vocalizations during the plastic song phase, between 45 and 75 dph, suggesting that this part of development is the range of the sensitive period when most vocalizations are being memorized and thus auditory memories are best formed (Johnson et al., 2002). Birds that produce more vocalizations during this plastic song phase end up with a stronger similarity to the tutor song at the end of development (Johnson et al., 2002). When zebra finches reach adulthood, around 80-100 dph, their song becomes stable and stereotyped ('crystallized') and the number of vocalizations produced no longer correlates with the similarity to the tutor song (Johnson et al., 2002). For zebra finches, the sensitive period appears to end around sexual maturity, ~90 dph, as untutored adults are unable to acquire a stable song (Funabiki and Konishi, 2003).

Only one song is typically learned during development and it is sung for the rest of the zebra finch's life. While some species, like parrots, exhibit open-ended learning and are able to acquire new vocalizations throughout their lifetimes, zebra finches exhibit age-limited learning; they are restricted to learning a song before sexual maturity (Brenowitz and Beecher, 2005). Thus, zebra finches, similarly to humans, have a sensitive or 'critical' period. The idea of the sensitive period does not exclude the possibility of learning another song, or in humans, language, later in life. While the capacity for humans and zebra finches to learn new languages and songs, respectively, decreases with age, both can learn new vocalizations later in development. For example, humans can learn second languages throughout their lives, but this process becomes more challenging and less successful later in life. Similarly, zebra finches can also learn song later in development (Yazaki-Sugiyama and Mooney, 2004). However, they are able to produce only one song during adulthood, unlike humans who can continue to speak multiple languages during adulthood. Zebra finches also deviate from humans as a model because they can become 'oversaturated' with song if exposed to excessive tutor song; there is a negative correlation between total amount of song exposure and song similarity to the tutor song during adulthood (Tchernichovski et al., 1999). For humans, excessive exposure to speech does not lead to degraded speech production. Furthermore, zebra finches raised with male siblings in addition to a male tutor for an extended period of time can produce poor and incomplete imitations; the more juvenile males there are in a clutch, the more dissimilar each of their songs are to that of the male tutor, usually the father, partially because the juveniles begin incorporating syllables and other elements from each other into their own songs (Tchernichovski and Nottebohm, 1998). Moreover, the eldest male has the highest song similarity to the tutor song during adulthood, with similarity decreasing in males born subsequently in that clutch (Tchernichovski and Nottebohm, 1998). All of these

findings each comprise one aspect of how to best optimize song learning in a laboratory setting: by implementing a short period with live one-on-one tutoring, we can maximize song learning during zebra finch development. The opportunity to manipulate the zebra finch's auditory environment results in an animal model in which vocal learning can be observed in detail.

***Anatomical Similarities*:**

In addition to developmental similarities, there are anatomically analogous regions between the human and zebra finch brains involved in processing species-specific sounds. Parallels between human auditory and higher-level speech processing areas and regions in the zebra finch brain are based on structural and functional similarities (Doupe and Kuhl, 1999; Mooney, 2009). Most notable are the analogies between Broca's area in the human brain and HVC in the zebra finch brain, which both participate in the production of sounds, and Wernicke's area in humans and the zebra finch caudomedial neopallidum (NCM), which are involved in auditory processing of sounds (Figure 3). The zebra finch midbrain auditory center (mesencephalicus lateralis pars dorsalis [MLd]), the analog of the human inferior colliculus, is also involved in auditory processing and has been implicated in song learning (van der Kant, 2015; van der Kant et al., 2013; Logerot et al., 2011) (Figure 4).

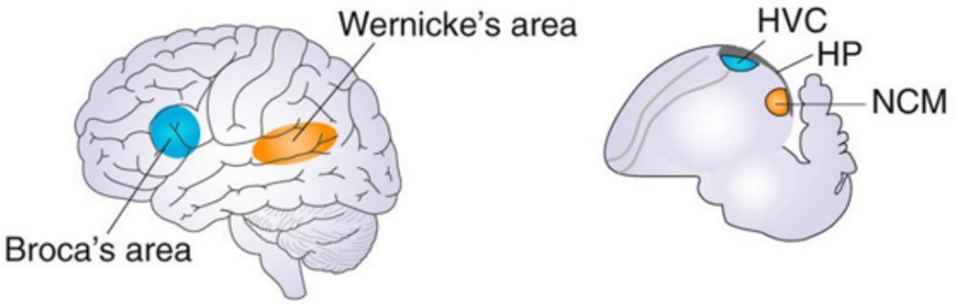


**Figure 3: Human language and zebra finch song processing areas.** Broca's area, highlighted in blue, in humans (left) is an area in the left temporal lobe involved with speech production, similar to HVC in zebra finches (right). Wernicke's area is an area in the human left temporal lobe, orange, involved with speech comprehension, similar to NCM in zebra finches. Figure taken from Moorman et al., 2015.

The circuit involved in perception and production of song in zebra finches, similar to the human speech pathway, has two branches: the auditory pathway and the song production (or 'motor') pathway. Literature suggests that both the auditory and the motor pathway are involved in learning and memory of tutor song (Ölveczky et al., 2005; Roberts et al., 2012). In the auditory pathway, input first passes through the midbrain nucleus MLd, then ascends through the thalamus into Field L, the zebra finch analog of human primary auditory cortex. Input from Field L then travels to auditory association areas such as NCM and CM (Figure 4). This thesis will focus on the contributions of the auditory pathway in song learning, rather than the motor pathway, as we are observing neural activation to listening to song, rather than its production.

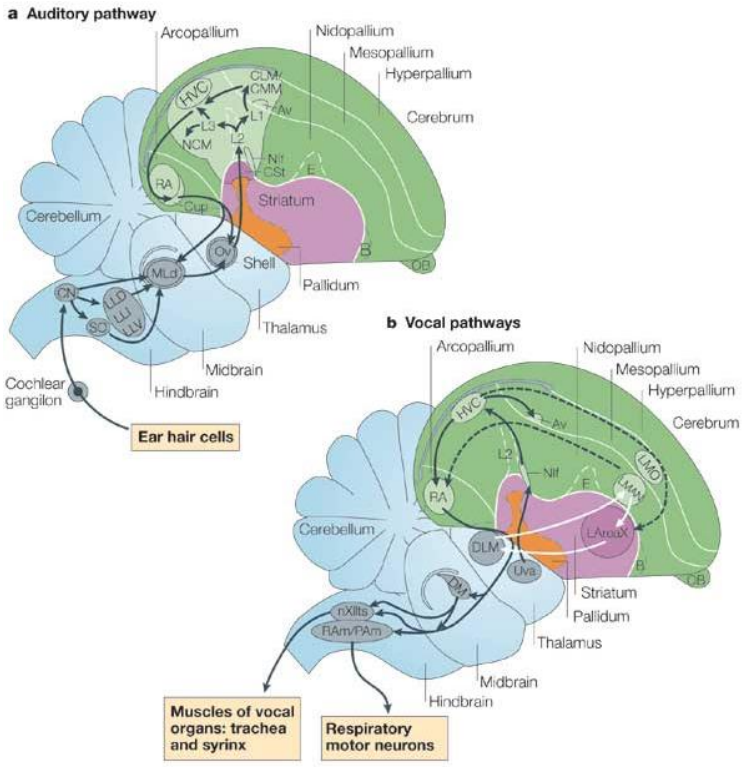


**Figure 4: Auditory and vocal pathways of the zebra finch brain.** a. Auditory input ascends through the zebra finch brain; beginning in hindbrain and midbrain nuclei, input then travels up into primary auditory cortex and then secondary auditory regions. b. The descending motor projections involved in song production originate in HVC to innervate RA, with various projections and inputs from other centers in the brain, leading downstream to vocal muscles. Figure taken from Jarvis et al., 2005.

There is a substantial body of evidence surrounding the role of the NCM in song learning and tutor song selectivity, the differential activation of tutor song compared to other conspecific songs (Gobes and Bolhuis, 2007; Woolley, 2012). NCM was first implicated in song learning during the 1990s using the immediate early gene (IEG) ZENK.[1] In the NCM, IEG expression upon re-exposure to the tutor's song is correlated with song learning Birds that learned more, measured as songs with high similarity to tutor song, showed higher IEG expression in the NCM. However,

[1] ZENK , also known as zif-258, egr-1, NGFI-A, Krox-24, is a transcriptional factor regulator of immediate early gene (IEG) established in zebra finch research as a neuronal marker for song learning as it is rapidly activated by auditory playback of song (Mello and Clayton, 1994; Mello et al., 1992). About 30 minutes after same species song exposure, ZENK mRNA levels peak in auditory structures such as Field L, NCM, CM, HVC and RA (Mello and Clayton, 1994). ZENK is used as indicator of neuronal changes associated with song-related memories.

IEG expression is not correlated with song similarity in other regions of the brain (Bolhuis et al., 2010; Terpstra et al., 2004). Thus, these findings, among others, posit that the NCM holds part of the neural representation for tutor song (Gobes and Bolhuis, 2007; London and Clayton, 2008). During the sensorimotor period, juvenile zebra finches have the greatest IEG activation to tutor song exposure in the NCM, compared to conspecific song and silence (Gobes et al., 2010). Like humans, juvenile zebra finches show left dominance for tutor song memory in the Wernicke-like region, NCM, suggesting that the NCM is involved in song learning and retaining the memory for the tutor song (Doupe and Kuhl, 1999; Moorman et al., 2012). Tutor song memory is maintained in the NCM through development and into adulthood, even when a bird is exposed to tutor song for a short period of time early in development (Phan et al., 2006). Left dominant neuronal activation to tutor and novel songs is found in HVC, the Broca-like region in the zebra finch brain, both in juveniles during the sensorimotor period and in adult birds (Moorman et al., 2012; Roberts et al., 2012). This suggests the neural representation for tutor song is not stored in one place alone, but rather distributed among a variety of nuclei.

While IEGs have been a useful tool in uncovering neural correlates of song learning, it is a limited technique as it allows for cross sectional but not longitudinal insights about development of song. IEGs do not allow for observation of neural activity at multiple points during development in the same subject. Functional magnetic resonance imaging, however, can be used to study the acquisition of model vocalizations noninvasively and thus allows for longitudinal study of song learning.

***Functional magnetic resonance imaging and songbirds:***

Functional magnetic resonance imaging (fMRI) is a noninvasive neuroimaging technique that allows us to observe neural activity using blood oxygenation as an indirect measure of neuronal responses (Logothetis and Pfeuffer, 2004). fMRI allows for the spatial visualization of the hemodynamic response to a stimulus, known as the Blood Oxygen Level Dependent (BOLD) response. Because fMR images are acquired quickly, images reflect the localized hemoglobin deoxygenation that occurs when neurons respond to a stimulus. Early work demonstrates that BOLD signal change occurs with a slight delay with respect to stimulus onset; the hemodynamic response observed using BOLD fMRI is not completely synchronous with neuronal activation, but with 1-2 second delay (Logothetis, 2003). Previous studies show that zebra finches, similar to humans, have auditory-evoked hemodynamic response times of 7-8 seconds, further establishing the link between auditory neuroimaging studies in humans and songbirds (Van Meir et al., 2005).

Unlike other techniques, such as electrophysiology and immunocytochemisty, which are typically used in animal research, fMRI possesses the unique benefit of being able to noninvasively observe a whole brain response to a stimulus in real time. While other techniques can observe cellular and molecular changes, they are usually terminal, focus on small regions in the brain, and can provide no information about neural changes that occur throughout development in a single subject. Noninvasive imaging techniques such as diffuse optical imaging (DOI) and fMRI provide novel and unique ways to observe brain responses to stimuli with relatively high temporal and spatial resolution, and with the added benefit of being able to observe these responses over time in a single subject (Lee et al., 2013; Van Ruijssevelt et al., 2013a). Additionally, because it allows for global brain response, we are better able to explore responses in multiple regions of the brain

within one experiment. Here, we take advantage of this technique to observe neural changes resulting from increased auditory experience over the course of development.

One limitation of fMRI lies with the behavioral state of the subject. In human imaging studies, subjects are usually awake. However, zebra finches are typically anaesthetized during imaging. Thus, fMRI can only inform us of what is happening in the anesthetized zebra finch brain. In addition, different anesthetics can induce different states of unconsciousness, which can bias results. A previous study addressed this issue in zebra finches, comparing the neural activity and BOLD response under three different anesthetics: medetomidine[2], an anesthetic previously used in bird studies, urethane[3], a popular small animal anesthetic with few effects on normal neural activity, and isoflurane[4], the most common clinical and large animal imaging anesthetic (Boumans et al., 2007). Boumans et al. (2007) concluded that there were two main differences. First, the average signal strength was greatest when using isoflurane, followed by medetomidine, and finally, urethane. Second, there was also larger neuronal activation by area under isoflurane and urethane, compared to medetomidine. Under isoflurane, the greatest number of significantly active pixels were found in primary auditory regions as well as other regions such as the NCM and CMM, followed by urethane and finally, medetomidine. Additionally, there was an interaction between anesthetic and stimulus studied. However, this study did not compare neural activity and BOLD response under anesthetic with that of awake birds, and therefore we do not know the extent to

[2] Medetomidine is a $\alpha_2$-adrenoreceptor agonist that produces sedation and analgesia.
[3] Urethane modulates several neurotransmitter-ion gated channels, increasing inhibitory effects of GABAergic and glycinergic synapses and decreasing excitatory effects of glutamatergic synapses (Boumans et al., 2007). Previous research suggested that urethane does not significantly affect song discrimination and spectral tuning during electrophysiology experiments, only affecting intrinsic neural excitability (Schumacher et al., 2011). However, urethane is mostly used for terminal experiments and thus, cannot be used for longitudinal experiments such as with fMRI (Van Ruijssevelt et al., 2013b).
[4] Isoflurane increases inhibitory effects of GABAergic synapses and slightly decreases excitatory effects of glutamatergic synapses (Boumans et al., 2007).

which results found under anesthetic state would differ from an awake state. Use of isoflurane is preferred in the field because auditory processing remains active under anesthesia. The alternative, animal training and habituation to the MRI environment, is time intensive and more technically challenging because motion artifacts generated by awake subjects are difficult to remove during post-processing (Van Ruijssevelt et al., 2013b). Use of anesthesia may be to our advantage because while some auditory nuclei, like primary auditory region, Field L, respond equally to song playback during awake and anaesthetized states, other nuclei, such as HVC, are not responsive in awake subjects, but are under anesthesia (Schmidt and Konishi, 1998). In awake birds, there is typically no response elicited by bird's own song playback in HVC, but under low doses of anesthetic, there is a neural response due to song playback, which suggests that anesthetics disinhibit neurons that are inhibited during awake states (Schmidt and Konishi, 1998). Such auditory "gating" has been similarly reported in Nif, a region upstream of HVC as well as RA and LMAN, regions that are downstream of HVC, during sleep and anesthesia (Konishi, 2004). Additionally in consideration of the use of anesthetics, we must also acknowledge that previous fMRI results have validated and replicated results obtained with IEG and electrophysiological techniques in awake birds (Boumans et al., 2008a; Van Ruijssevelt et al., 2013b). Thus, while we are not able to definitively address the neuronal response during awake auditory processing, using anesthetics still provides valuable and reliable data about auditory processing of song.

While fMRI is now commonly used in humans, only relatively recently was this method adapted and optimized for small brains, such as that of the zebra finch, with enough spatial and temporal resolution to address biological questions about vocal learning (Van der Linden et al., 2009). Because of the air pockets in the zebra finch skull, traditional methods and imaging sequences, in particular Echo Planar Imaging (EPI) or Gradient Echo (GE or FLASH), used for

humans and neuroimaging of mammalian model organisms, do not provide enough resolution in the avian brain to conduct biological experiments without extensive post-acquisition processing. New imaging protocols using Spin Echo sequences which are less sensitive to in-field inhomogeneity have been developed successfully (Van der Linden et al., 2009; Poirier and Van der Linden, 2011; Van Ruijssevelt et al., 2013a). Spin Echo provides a major improvement to other pulse sequences because it maintains undistorted functional images in multiple slices in the same amount of time that another pulse sequence could provide images of one or two slices (Van Ruijssevelt et al., 2013). The first demonstration of this method in zebra finches showed that the hemodynamic response to an auditory stimulus was similar to what had been observed in humans, making it a viable tool for biological experiments (Van Meir et al., 2005). In zebra finches, fMRI has been shown to both corroborate and add to previous IEG and electrophysiological experiment results regarding neural response to song in various auditory regions.

Experiments in adult zebra finches that implement fMRI have shown that several song nuclei, including Area X, Field L, HVC and MLd (Figure 4), are implicated in bird's own song (BOS) and conspecific (CON) song perception and memory (Poirier et al., 2009). The response to BOS, compared to conspecific song, is right lateralized in Area X, Field L and HVC, with highest response amplitude in Area X; the response to CON, compared to heterospecific song (HET), song from another species of bird, is left lateralized in these nuclei, but the greatest responses is elicited in Field L (Poirier et al., 2009) (Figures 5 & 6). However, it is unclear whether Field L is really selective for CON. It was previously found that Field L, the NCM and CMM do not show stimulus specificity, a significant differential response to one stimulus only (BOS or CON), although there is still a preference for timing characteristics specific to zebra finch song in these regions (Boumans et al., 2007, 2008b). It has been postulated that there is a hierarchy of the subregions

within Field L based on BOLD signal strength, where L2a and L2b show no BOS or CON specificity (Boumans et al., 2008a). In addition, Field L is more readily activated by song stimuli than the NCM and ventral Field L seems to be sensitive to preservation of both temporal and spectral aspects of song (Boumans et al., 2007).

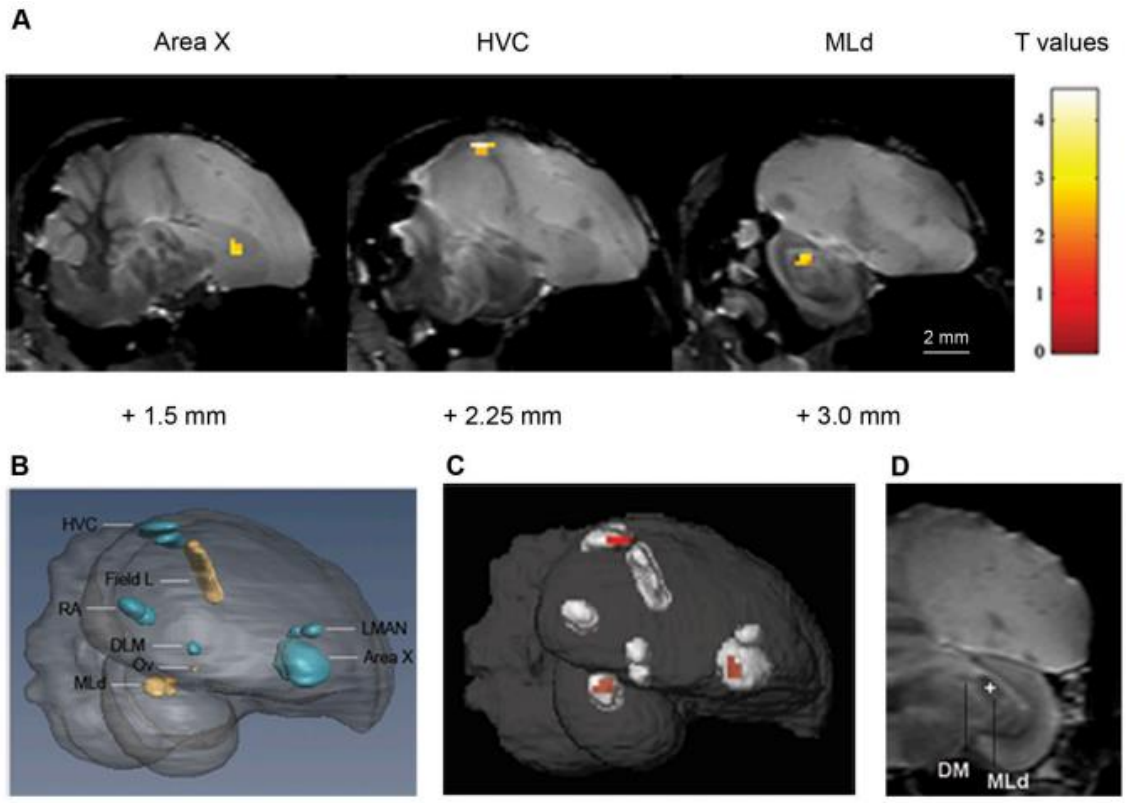


**Figure 5: Selective neural response to bird's own song.** A. Own song specificity, compared to conspecific song, found in right Area X, HVC and MLd in adult zebra finches. B. Rendering of relevant song nuclei using anatomical MRI in sagittal view. Field L and MLd are highlighted in yellow. C. Activation in A overlaid onto rendering (B) is shown. D. Midbrain nucleus, MLd (+ in image), can be positively identified as a separate nucleus from neighboring DM in coronal sections. Figure taken from Poirier et al. 2009.

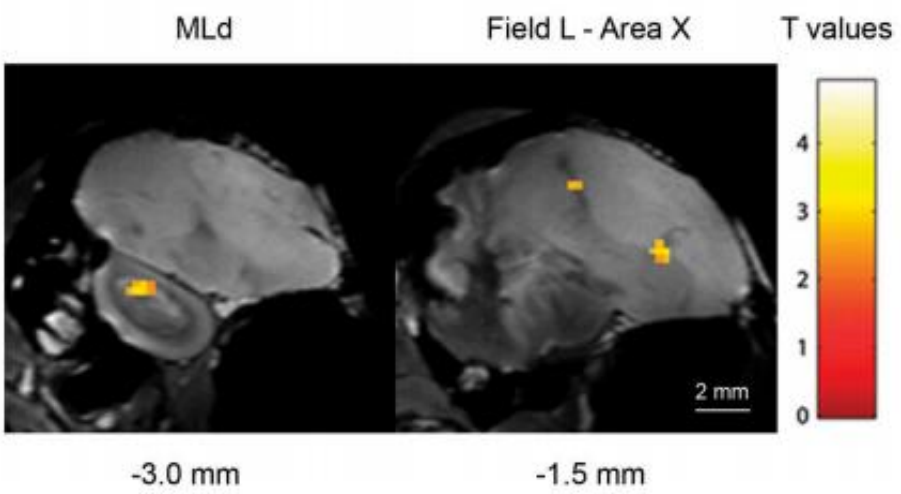


**Figure 6: Selective neural response to conspecific song.** Greater activation to conspecific song compared to heterospecific song, found in left MLd, Field L and Area X. Figure adapted from Poirier et al. 2009.

Since a number of studies using IEGs have shown evidence for the NCM involvement in song learning and memory, observing activation in the NCM using fMRI to study stimulus specificity is a logical follow-up (Bolhuis et al., 2000, 2001; Voss et al., 2007). However, studies with a focus on the NCM are less prevalent in fMRI research, partially because while the NCM is significantly activated by each separate stimulus, BOS, CON and tutor song (TUT), there are often no differential activations and no correlations between amplitude of activation and song learning found in juvenile or adult zebra finches (van der Kant, 2015; van der Kant et al., 2013). Using fMRI, the NCM has been found to be involved with recognition of song features; the less white noise and more song elements, such as syllables, calls or whole motifs, a stimulus has, the more the NCM is activated by the stimulus (Boumans et al., 2008a).

More recently, fMRI studies in zebra finches have focused on the auditory midbrain nucleus, MLd, in order to observe its role in tutor and own song specificity during development and adulthood. Previous research on the zebra finch auditory midbrain was restricted to questions about song and tone discrimination (Woolley, 2012). Using fMRI, however, MLd has been

implicated in exhibiting selectivity for copied vocalizations (van der Kant et al., 2013). BOS, TUT and CON all significantly activate the nucleus, with BOS and TUT each eliciting greater response than CON (van der Kant et al., 2013). A significant correlation between BOLD signal amplitude in response to TUT or BOS and learning strength, as measured by tutor-tutee song similarity, was found (van der Kant et al., 2013). This provides evidence for the hypothesis that MLd also may be part of a distributed neural substrate for tutor song memory.

Presently, there is only one study observing zebra finch song development longitudinally using fMRI. A 2015 doctoral thesis specifically looked at the activation and song specificity of MLd throughout development (van der Kant, 2015). Birds that underwent normal tutoring during development were imaged at 30, 40, 60, 100 and 500 dph to observe changes in responsiveness to TUT, CON and HET. Van der Kant (2015) shows that there is no TUT specificity in MLd at 30 and 40 dph, during the birds' sensory phase, but that there is an overall peak in activation to CON, which indicates the end of the sensory phase. This adds to the evidence that, at this age, male birds are receptive to adult male song, but have no neuronal preference for a specific song yet. Further, at 60 dph, during the sensorimotor phase when tutees begin to practice vocalizations, there is a strong tutor-song selective response seen in left MLd. After that, tutor song selectivity wanes and by 100 dph, by which time tutees typically sing a final crystallized song, there is no tutor song selective response in left or right MLd. Tutor song activation, however, remains high through adulthood. While MLd consistently responds to tutor stimuli compared to silence, it does not show greater activation towards tutor song compared to a conspecific song consistently throughout development and adulthood. Van der Kant also found that there was a trend towards tutor song selectivity in right MLd during adulthood, but this was inconclusive and the effect observed was variable depending on the individual bird. In addition, during development, there is little neural

discrimination between conspecific and heterospecific song, but in adulthood, right MLd shows greater preference for conspecific song over heterospecific song. These results reflect that the response to tutor song is plastic during development, and the response to tutor song remains elevated into adulthood while activation resulting from conspecific song decreases. This suggests that because tutor song is more salient, and remains relevant to the tutee for longer, there is greater retention and potentially greater activation to the tutor song. Tutor song activation reaches peak amplitude at the time when a juvenile bird is learning and practicing song, at 60 dph, and wanes afterward, when song is crystallized, and the tutor song memory is no longer needed to produce the desired behavior. This further supports the notion that MLd holds at least part of the neural substrate for tutor song memory.

Although little research has been conducted observing the neural responses of socially isolated or untutored zebra finches using fMRI, the evidence available suggests that socially isolated birds (who receive no tutoring) have differential neuronal responses to various aspects of song compared to normally tutored birds (Maul et al., 2010). Compared to normally tutored males, box trained males, who received 20 song playbacks per day, and completely isolated males both experienced a broader distribution of Field L activation response to song stimuli (Maul et al., 2010). In addition, social isolation led to lack of stimulus specificity; while box trained and normally tutored adults showed a difference in activation of Field L between conspecific song, bird's own song, and tutor song, socially isolated birds did not (Maul et al., 2010). This leads to two conclusions: 1) auditory deprivation changes spatial activation of Field L, and 2) early auditory experience is needed, not only for vocal production, but also for auditory discrimination responses.

Previous fMRI research in zebra finches has shown that multiple auditory areas can be activated by the playback of various stimuli and that different areas of the zebra finch brain respond

to different aspects of song as well as different songs, during development and into adulthood (van der Kant, 2015; van der Kant et al., 2013). A neural representation of the tutor song remains through development and into adulthood, long after the tutoring period has ended (van der Kant, 2015). However, we do not fully understand how auditory experience during development changes the underlying neural substrate for tutor song memory. In this study, we investigate activation of auditory nuclei after a short tutoring period during the sensorimotor phase of development. By observing the neural response to tutor song at multiple points in time, we can determine how neural activation to tutor and conspecific songs changes during development with respect to a juvenile zebra finch learning the tutor song. We hypothesize that a high response to tutor song in MLd, and other auditory nuclei, after tutoring will be evident and may be related to high levels of song learning, thus discerning the relationship between how well a juvenile bird can learn during the sensorimotor period and the neural response to the target song.

# Methods

### *Subjects and subject maintenance*

Male zebra finches (*Taeniopygia gutatta*) (N = 15) were bred and housed at the Wellesley College animal facility. Subjects were housed in a 16 hour light/ 8 hour dark cycle with food and water *ad libitum*. Subjects whose tutors did not sing during the five-day exposure period were excluded from all analysis.

### *Experimental timeline*

Subjects were separated from their father at 7 days post hatch (dph), and housed with their mother and siblings in a house-made sound-attenuating chamber. At 32 dph, subjects were isolated from their mother and the other siblings in the clutch and housed individually in acoustic isolation. Starting at 49 ± 1 dph, each subject received live tutoring with an adult male tutor for five consecutive days. During the same day, right before the tutor was introduced to the subject and two days after the tutor was removed, subjects were imaged using MRI. During adulthood, after birds achieved crystallized and stereotyped adult song, one follow-up MR imaging session was conducted (120 ± 1 dph, Figure 7).

Each subject had a different tutor that was not biologically related to the subject.

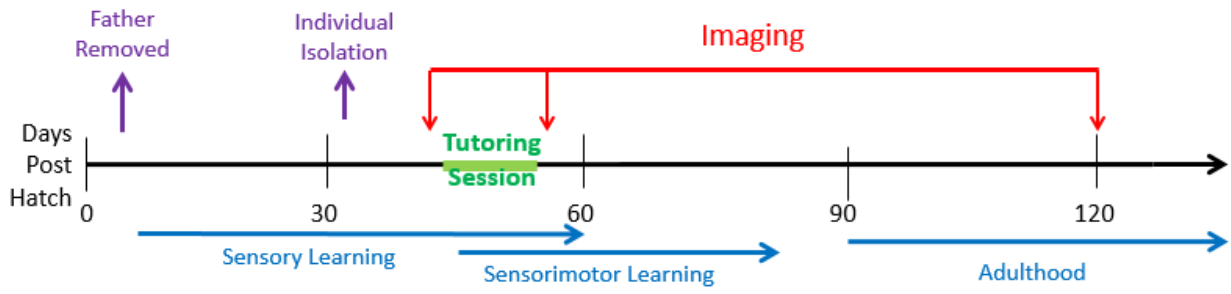


**Figure 7: Experimental Timeline**

***Sound recording***

Subjects were individually housed in sound isolation boxes until the conclusion of the experiment. Song recording was achieved using an in-cage microphone (Shure SM 93) connected to NADI pra-8 microphone pre-amplifiers attached to a National Instruments PCI card and custom written software (Labview, National Instruments) and recorded at 44.1 kHz.

***Magnetic Resonance Imaging***

All MR images were acquired at 400 MHz in a 9.4 T Bruker Avance vertical wide bore NMR spectrometer with microimaging accessory (2.4 G/cm/A gradient strength) using Bruker Paravision 4.0 software.

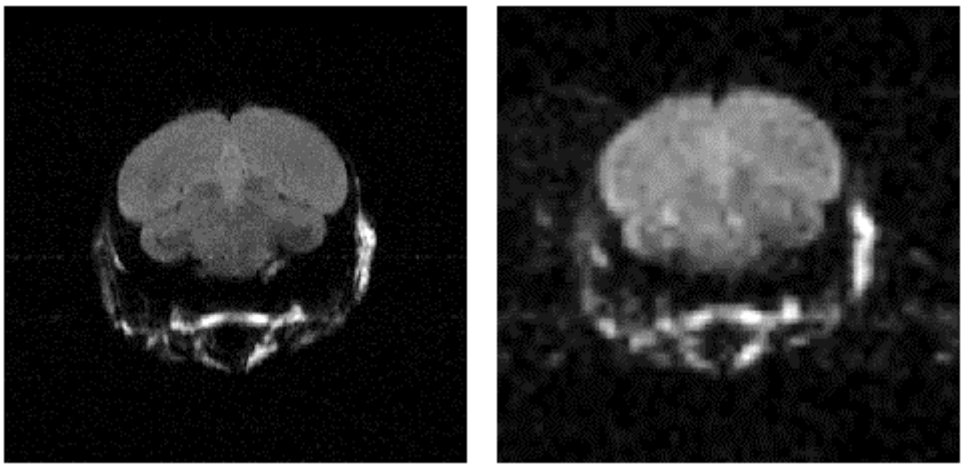

**Figure 8: Example slices of anatomical and functional scans.** Center coronal section taken from same subject from same imaging session. Acquisition time of anatomical scans (left) is much longer, 13 minutes, thus resulting in much more spatially resolved images (voxel size 0.0977 x 0.0977 x 0.75mm). Functional images (right) take only 16 seconds to acquire and thus are less spatially resolved but serve to be more temporally resolved (voxel size 0.39 x 0.39 x 0.75mm).

After shimming and tuning and matching, a scout RARE tripilot image was taken to observe the subject position in three orthogonal slices (TR: 2000 ms, TE: 12.5 ms, RARE factor: 8, acquisition matrix: 128x128). Fifteen contiguous coronal $T_2$ weighted anatomical slices were

acquired using a Spin Echo RARE sequence (TR: 3109 ms, TE: 60 ms, RARE factor: 8, acquisition matrix: 256x256, FOV: 2.50 x 2.50 cm, 13 minutes acquisition time). Functional images were acquired using a Spin Echo sequence with the same geometry as the anatomical images acquired (TR: 2000 ms, TE: 60 ms, RARE factor: 8, acquisition matrix: 64x64, FOV: 2.50 x 2.50cm). Imaging sequences also included fat and motion suppression. Examples of anatomical and functional image resolution are shown in Figure 8.

All functional images were queued using the Paravision Queued Acquisition Scanning tool and were consecutively acquired automatically. An approximately 2-5 second delay between functional scans in the queued scanning macro is devoted to shimming between scans.

Total acquisition time per session was about two hours. All subjects recovered immediately after imaging.

Protocol for imaging can be found in Appendix #1.

***Synchronous imaging and auditory playback***

Simultaneous imaging and auditory playback were achieved through signal relay between Neurobehavioral Systems (NBS) Presentation software and Paravision software. An Arduino Uno device was set up to interpret signal originating from Paravision and deliver it to the Presentation software (Arduino code courtesy of NBS [see Appendix #2]). At the beginning of every functional image sequence, the signal was produced, relayed and interpreted by Presentation with no significant time delay in auditory stimulus onset.

Stimulus delivery was cued by NBS Presentation software on a Lenovo Thinkpad Edge laptop and was delivered to subject's ear openings via a custom-made MRI compatible speaker (20 mm 8 Ω Digi-Key #102-1542-ND with magnet removed by hand). Stereo plug (3.5mm, MCM

Electronics, MCM #27-3146) and speaker were connected using plastic Molex connectors (.062" diameter, Digi-key #76650-0066) and shielded twisted pair wire, which was used to minimize the wire acting as an antenna. Connectors were used to easily disconnect the speaker half without removing it from the protective casing in the bird bed. Stimuli presentation code was written in house (See Appendix #2).

***Animal preparation during imaging***

Thirty minutes prior to imaging, food and water were removed from subjects' enclosures. Subjects were anesthetized using 2% isoflurane in $O_2$ (flow rate of 0.4 L/min) in a plexiglass chamber (Braintree Scientific, Braintree MA). Subjects were then positioned and secured into the custom-made acrylic bed (Figure 9). Respiration was monitored using a pneumatic pressure respiration sensor pad (Sims Graseby) placed underneath the subject's chest and BioTrig Builder 1.01 software on a Dell Latitude laptop. Respiration rate was maintained at 50-100 breaths per minute using 1.5% isoflurane/$O_2$. Isoflurane concentration was achieved using a VIP Veterinary Vaporizer (Colonial Medical Supply Co., Franconia, NH). Gas and exhaust were introduced into the head cone using plastic tubing; exhaust was connected to F/AIR Scavenger System activated charcoal filters (Paragonmed, Coral Springs, FL).

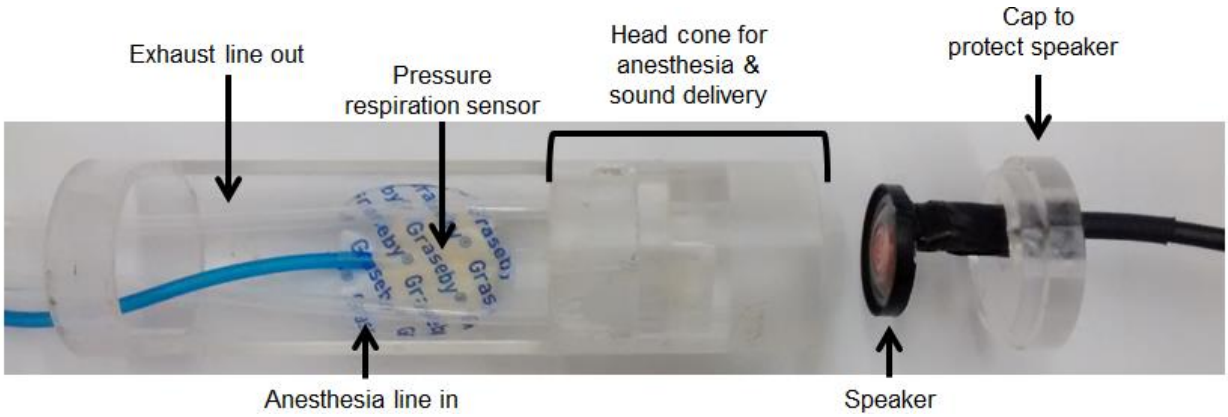


**Figure 9: Bird Bed.** Acrylic bed was custom made for zebra finch subjects. Anesthesia is delivered into the head cone by gas line in, and is cycled out of the chamber by gravity through the exhaust line out. Pressure respiration sensor is used to monitor subject respiration throughout experiment. Sound is delivered through custom-made MRI compatible speaker inserted into the head cone and secured by an acrylic cap. Sound travels through drilled holes in acrylic from the speaker chamber directly to the bird's ear openings.

The region of the magnet bore in which the bird was placed was maintained at 36° C during MR imaging, which was as close as technically possible to avian internal temperature of 42°C.

***Experimental paradigm***

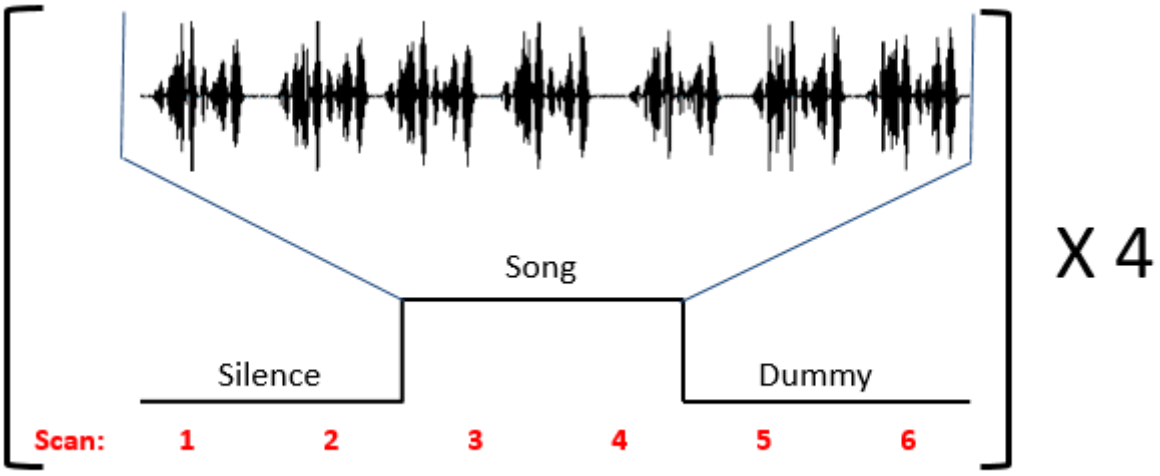


**Figure 10: Experimental Paradigm.** Each paradigm contained four blocks, each consisting of 2 scans, alternating between silence, song stimuli (shown as an amplitude-by-time spectrum above) and dummy scans that were devoid of stimulus and not used in further analysis.

A block paradigm, consisting of two fMR images per block, was used (Figure 10). Blocks of song were alternated with blocks of silence, which served as a baseline for activation potentially caused by noise of gradients during MR imaging. Additionally, two dummy scans were added after song blocks; the dummy scans were not used in data analysis but used as buffer between stimulus and silence image blocks. Each stimulus was presented four times, making each paradigm 24 images total.

***Auditory stimuli***

Conspecific and tutor song stimuli were created using PRAAT software. Tutor song stimuli were created from recordings made prior to tutoring. Conspecific song stimuli were created from adult male zebra finches that were not related to the subjects or tutors. Song similarity analysis using Sound Analysis Pro was done between conspecific and tutor song motifs to determine dissimilar conspecific songs in each experiment (<45% song similarity). Overall, there were no significant differences in % similarity among the three time points (Figure 11). Stimuli were created by taking one representative motif from one song bout and stringing motifs together, punctuated by silence between motifs (2 seconds total per stimulus), until the stimulus reached 15 seconds in length. Intensities (loudness) of all stimuli were equalized in terms of root mean square amplitude using a previously written PRAAT script to match intensity across all motifs and all stimuli. Additionally, equalizing RMS amplitude creates better discrimination between song motifs and silence or any background noise. Finally, stimuli were amplified 25% to be clearly audible inside the MR instrument during image acquisition. The protocol for stimuli creation can be found in Appendix #3.

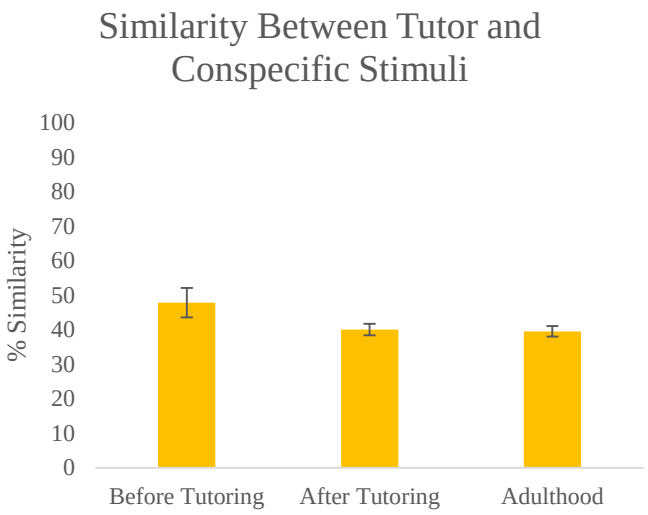


**Figure 11: Comparison of tutor and conspecific stimuli used during imaging.** Averages of % similarity between tutor and conspecific motifs used for each imaging session shown. Each subject had the same tutor song stimulus and a different conspecific song stimulus at each time point. No two subjects were presented with the same three conspecific stimuli in the same order. There are no significant differences in similarity between sets of stimuli used at each time point. Mean ± SEM shown.

***Behavioral data analysis***

To determine whether subjects learned significantly from their tutors, representative motifs from subjects were collected and compared to motifs from the tutor and another conspecific bird. Motifs for tutors were collected during adulthood, at no specific age. To compare whether juveniles learned specifically from the tutor or simply shared general characteristics with zebra finch song, motifs from adult conspecific birds from a separate colony were used (Moorman et al., 2012). Representative motifs from subjects were created from recordings from the same day as imaging sessions using PRAAT software. Subject motifs were collected between 10 am-12 pm for all time points and all subjects to eliminate any possible variability in song due to time of day. Song similarity analysis between tutor, conspecific and subject motifs was conducted using Song Analysis Pro 2011 (Tchernichovski et al., 2000).

***fMRI data analysis using SPM***

Statistic Parametric Mapping (SPM8, Wellcome Neuroimaging Center at University College London) was used to perform voxel based statistical analysis on all fMR images. Voxel sizes were enlarged to be more comparable to those of human imaging, so that SPM was better able to run analysis on the images. All image headers were opened individually and manually using the NIFTI toolbox for SPM, to increase voxel sizes by a factor of 10 in each dimension. Final voxel sizes were 0.977 x 0.977 x 7.5 $mm^3$ for anatomical images and 3.9 x 3.9 x 7.5 $mm^3$ for all functional images. Voxel sizes were adjusted to be uniform across all subjects and imaging sessions. The protocol can be found in Appendix #4. Subsequently, images were realigned to ensure spatial alignment among all images from one session. A general linear model reflecting the block paradigm used for auditory stimulation was specified and analyzed. A global brain analyses looking at all voxels within the entire brain for significantly different activation between tutor song and silence as well as conspecific song stimuli and silence were conducted. Protocol for global brain fMRI data analysis using SPM8 can be found in Appendix #5.

Regions of interest (ROIs) were defined manually for each subject and each imaging session by finding the coordinates for centers of target nuclei, NCM and MLd. ROIs were modeled as spheres with diameters 5mm (MLd) and 6mm (NCM). Centers were found by comparing the subject's anatomical image to the previously established MRI zebra finch atlas (Poirier et al., 2008). A list of center coordinates can be found in Appendix #6. ROIs were defined and built using Marsbar, a region of interest toolbox for SPM (Brett et al., 2002). Marsbar was used to extract the data and general linear model from global brain analysis of each imaging session. T statistics and percent signal change measures were calculated using Marsbar and recorded in Microsoft Excel. Protocol for ROI analysis can be found in Appendix #7.

***Statistical Analyses***

To assess whether juveniles learned specifically from their tutors, a repeated measure ANOVA with time (before tutoring, after tutoring and during adulthood) and comparison song (tutor or conspecific) as within-subject factors was conducted.

Region of interest measures were analyzed using paired t tests to test differences in neural activation between the two stimuli (TUT vs. CON) for each of the four ROIs and between the two hemispheres for the same region to the same stimulus (i.e. TUT response in left MLd vs. right MLd) for the two measures collected from ROI analysis: T statistic and percent signal change.

Due to technical difficulties, data from each bird was not available at every time point, but all birds were followed before and immediately after tutoring (n = 13) and most birds (n = 8) were followed successfully through adulthood. Updated results with data will all birds followed through adulthood is found in Appendix #8.

All repeated measure ANOVAs, paired t-tests and bivariate correlations were conducted using SPSS Statistics software package (IBM, Version 23). Graphs were generated using Microsoft Excel. Graphs show mean ± SEM, unless otherwise stated. Pearson's coefficient r is reported for correlations. Uncorrected p values are reported unless otherwise stated.

# Results

***Behavioral results***

Our results do not support that, overall, juveniles were able to learn significantly from their tutor as compared to novel non-tutor conspecific birds after five days of live social tutoring. There was no overall main effect of time (repeated measures ANOVA: $F_{(2,12)} = 1.917$, $p > .05$, $n = 7$), but there was a significant main effect of comparison song, (repeated measures ANOVA: $F_{(1,6)} = 8.330$, $p < .05$), suggesting that, over time, the % similarity did not change for each comparison song, but % similarity between conspecific and tutor songs was significantly different. A trend towards a significant interaction between time and song comparison was found (repeated measures ANOVA: $F_{(2,12)} = 4.340$, $p = .08$, n.s.). Paired t tests between tutor and conspecific similarity at each time point were conducted and showed there was a significant difference between similarity with the tutor song (78 ± 12.5% average similarity) as compared to similarity with a conspecific song (39 ± 15% similarity) during adulthood (paired t test: $n = 7$, $p < .001$, Figure 12), as well as before tutoring (paired t test: $n = 12$, $p < .05$) and immediately after tutoring (paired t test: $n = 12$, $p < .05$). There was a trend towards a significant difference between tutor song similarity before tutoring and during adulthood (paired t test: $n = 7$, $p = .07$, n.s.).

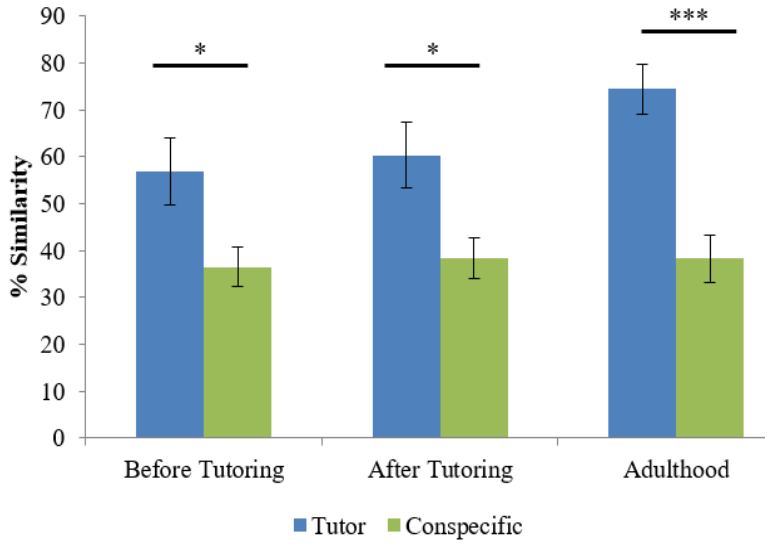


**Figure 12: Similarity between subjects and tutor and conspecific songs over time.** Similarity of subject song is not different over time in comparison to either tutor or conspecific songs. Similarity is significantly different between subject song and tutor and conspecific songs at any time point. A trend in increasing similarity to the tutor song was observed when similarity before tutoring was compared with similarity in adulthood. Mean ± SEM shown; before tutoring: n = 12, after tutoring, n = 12, adulthood: n = 7.

* $p < .05$

*** $p < .001$

### *fMR Imaging Results*

First, a global brain analysis looking for significant clusters in the entire brain was conducted for each subject at each time point. Because of variation in spatial geometries of imaging sessions, there was no adequate way to conduct group analysis on global brain activation, so we performed Region of Interest (ROI) analysis to observe activation specifically in two auditory nuclei: bilaterally in both MLd and NCM. ROIs were modeled as spheres with centers determined for each individual bird and imaging session separately (Figure 13, see Appendix #6 for full list of center coordinates used).

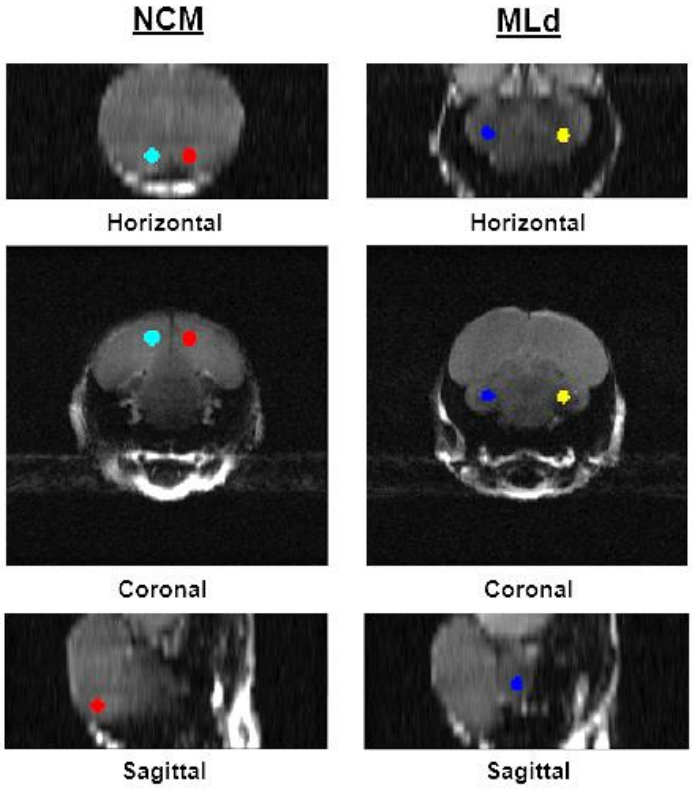


**Figure 13: Representations of defined Regions of Interest** Regions of interest (ROI) for NCM and MLd were defined for each imaging session and bird individually. ROIs were built as individual spheres with standard diameters with centers defined by coordinates on each subject's anatomical images, compared to the zebra finch fMRI atlas (Poirer et al., 2008). Representative ROI overlays on horizontal, coronal and sagittal views of one adult subject's anatomical are shown. Views originate at center coordinates of ROI.

(Light blue = left NCM, red = right NCM, dark blue = left MLd, yellow = right MLd)

Because of the geometric constraints there were few ways to determine and quantify the spatial variation between birds and sessions. One measure that was possible to take is the volume of the ROIs. ROIs made for left and right MLd did not significantly differ in volume (after multiplying the actual image dimensions by 10: left MLd: 529.8 ± 71.5 $mm^3$, right MLd: 525.9 ± 22.1 $mm^3$, $p > .05$, n.s.); neither did ROIs made for the left and right NCM (left NCM: 907.2 ± 25.5 $mm^3$, right NCM: 905.5 ± 21.6 $mm^3$, $p>.05$, n.s.). Thus, any further results found using ROI analysis were not confounded by maximum activation possible per volume, but were due solely to biological phenomena.

To observe how activation to tutor and conspecific song change in MLd and NCM over time we conducted paired t tests to look at differences in neural activation between stimuli (TUT vs. CON) in each of the four ROIs constructed and between hemispheres (left vs right) in MLd

and NCM at each time point for two measures derived from ROI data: T statistic of contrast and % signal change. T statistic of contrast refers to a measure of statistical testing performed by SPM that calculates the difference between voxels in one condition (stimulus) and another (silence), known as the contrast value, and divides it by the standard error of the contrast. Percent (%) signal change is a measure of signal intensity within each voxel and region and observes the effect of the stimulus. We hypothesized that immediately after tutoring and during adulthood there would be differences in these two measures in MLd and NCM between tutor and conspecific song stimuli, whereas no differences would be observed before tutoring.

We first measured the average T statistic of activation in MLd and NCM for each time point and stimulus (Figure 14). Before and immediately after tutoring, there were no significant differences between hemispheres, regions and stimuli. However, during adulthood, there was a significant difference between left and right MLd at 120 dph in response to TUT (left: 0.80 ± 0.33 SEM, right: -0.66 ± 0.33 SEM, paired t test: $n = 6$, $p = .01$, Figure 14) and between TUT and CON in right MLd (TUT: -0.66 ± 0.33 SEM, CON: 0.23 ± 0.27 SEM, paired t test: $n = 6$, $p = .05$, Figure 14). This suggests that right MLd responds less to tutor song than to conspecific song but it also is less activated by tutor song than left MLd. No significant results were found in the NCM for the average T statistic of activation.

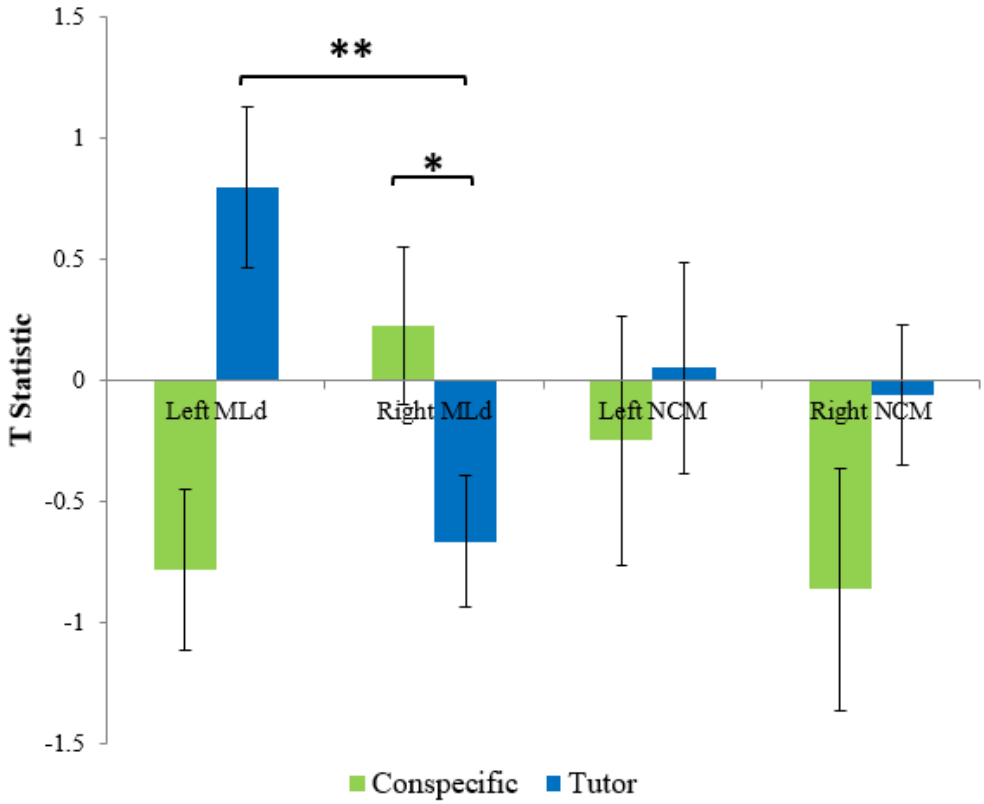


**Figure 14: Mean ± SEM of T Statistic of each ROI at Adulthood.** T statistic of each ROI was determined in response to tutor and conspecific song at adulthood. Data shown for all birds ( n = 6 ).
* p < .05, ** p < .001

Percent signal change, another measure of neural activity observing changes in signal intensity within a region, was also calculated (Figure 15). Similar to the T statistic, there were no significant differences between regions, hemispheres and stimuli before or immediately after tutoring. However, again during adulthood, there was a significant difference between left and right NCM in response to CON (left: -1.06 ± 3.24 SEM, right: -6.64 ± 3.38 SEM, paired t test: n = 6, p = <.05, Figure 15).

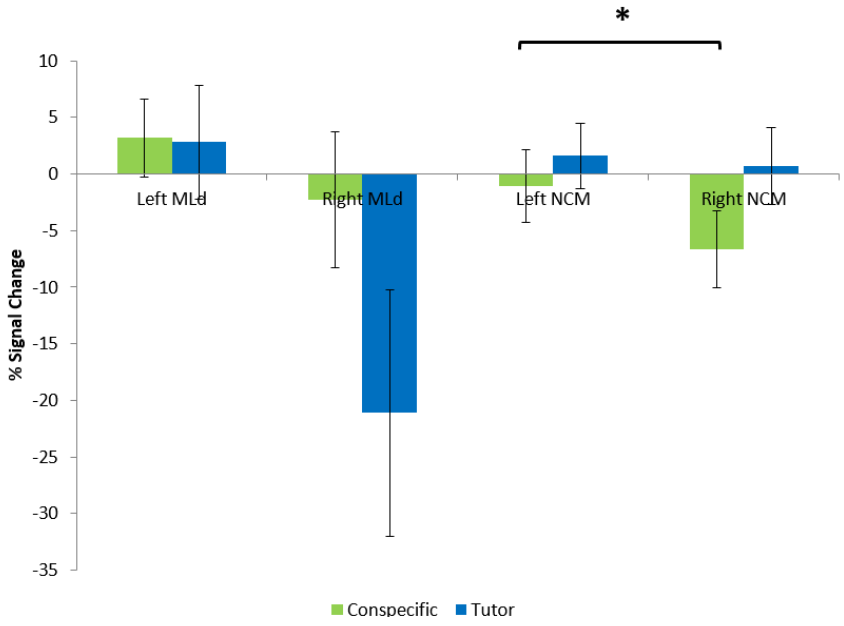


**Figure 15: Mean ± SEM of % Signal Change of each ROI at Adulthood.** % signal change of each ROI was determined in response to tutor and conspecific song at adulthood. Data shown for all birds ( n = 6 ).
* p < .05

We then wanted to determine if there was a relationship between how well a bird is able to imitate a tutor song and the neural responses to tutor and conspecific songs during development, so we conducted bivariate linear correlations between tutor song similarity at adulthood and neural measures in the four regions at every time point. We hypothesized that stronger neural activation to tutor song in any region immediately after tutoring would be positively correlated with higher learning scores, thus neural activation could predict learning outcome. Based on previous research, we also hypothesized that strong activations during adulthood to tutor song would be correlated with learning strength.

A positive correlation in left MLd between response to tutor song before tutoring and tutor song similarity at adulthood was found in both % signal change and T statistic measures (% signal change: r = 0.806, n = 7, p < .05, T statistic: 0.840, n = 7, p < .05, Figure 16). This suggests that

birds that had a stronger activation to the tutor song—the first time they heard it— would learn the song better. However, there were no significant correlations between % signal change in response to tutor song immediately after tutoring and tutor song similarity at adulthood in any region, thus we did not find that activation after tutoring predicted learning outcome.

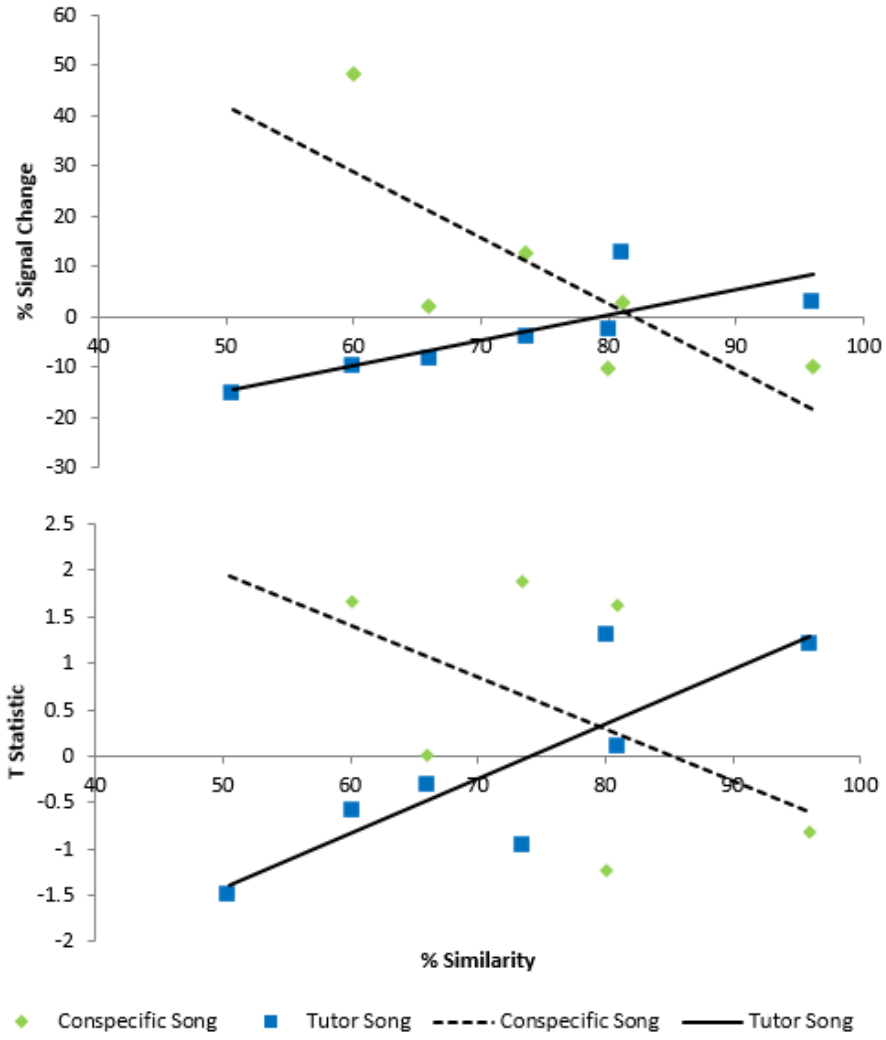


**Figure 16: Correlations between T Statistic and % signal change in left MLd before tutoring and tutor song similarity during adulthood.** T statistic and % signal change were both positively correlated with similarity to tutor song at adulthood (% signal change: $r = 0.806$, $p < .05$, T statistic: 0.840, $p < .05$). Correlations between tutor song similarity and response to conspecific song were not significant. Data shown for all birds ( $n = 7$ ).

Furthermore, there were positive correlations between % signal change in response to TUT in both left and right NCM and TUT similarity during adulthood (left: r =.836, n = 6, p < .05, right: r = .845, n = 6, p < .05, Figure 17). The stronger the activation in either of these two regions in response to tutor song, the better the bird had copied its tutor's song.

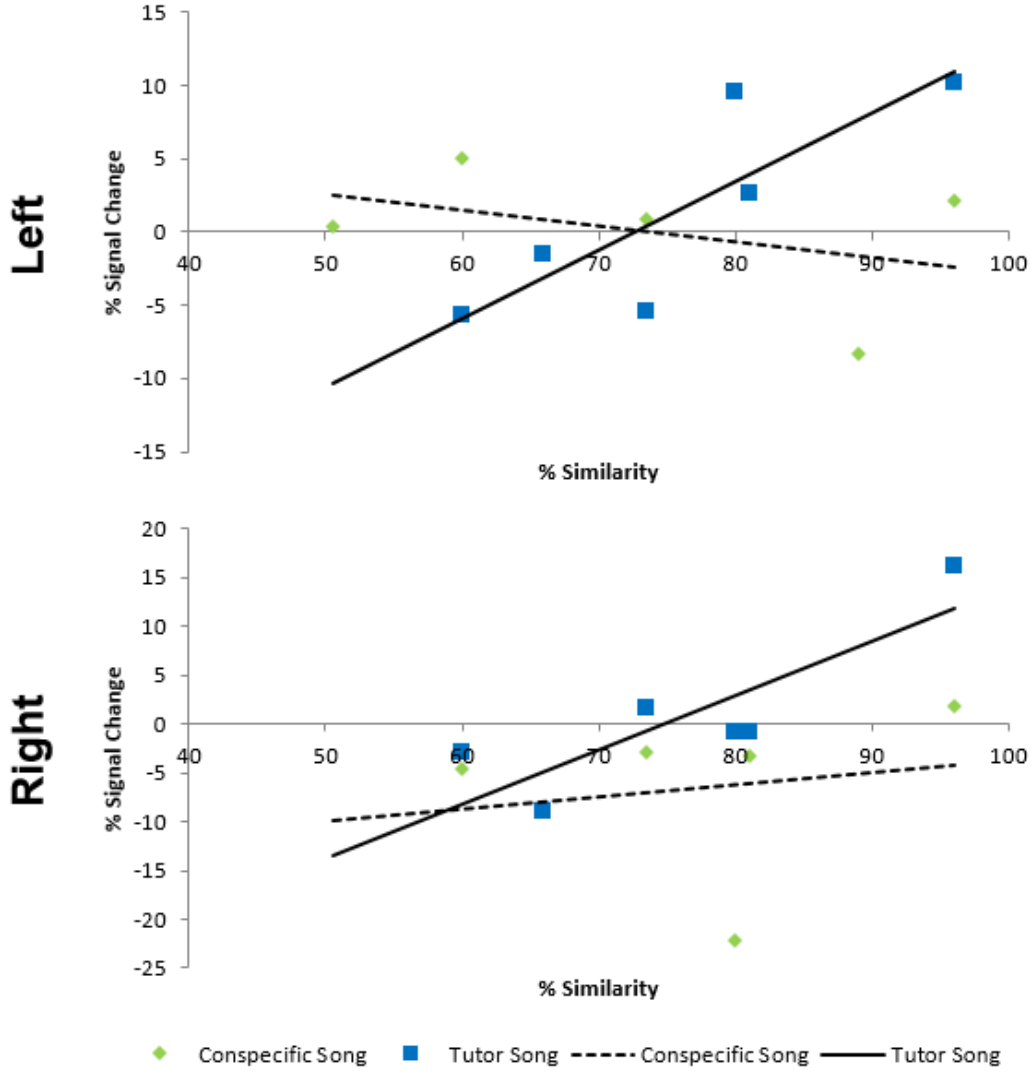


**Figure 17: Correlations between % signal change in the left and right NCM at adulthood and tutor song similarity at adulthood.** % signal change was positively correlated in both the left and right NCM in response to tutor song at adulthood with similarity to tutor song at adulthood (left: r =.836, p < .05, right: r = .845, p < .05). Correlations between % signal change in each region in response to conspecific song were not significant. Data shown for all birds (n = 6).

# Discussion

In this study, we focus on neural responses as a direct result of learning a tutor song during development and in adulthood while previous fMRI studies have only focused on the development of neural responses to various songs in normally reared juvenile and adult zebra finches. This is the first study to investigate neural activation in auditory nuclei before tutoring, when a juvenile hears song for the first time, immediately after a short tutoring experience and during adulthood, using fMRI. We observed the development of neural responses to tutor and novel conspecific songs that develop and persist due to learning a tutor song longitudinally in zebra finch subjects. Additionally, we investigate how these neural responses to song stimuli relate to a bird's ability to successfully imitate a tutor song.

### *Zebra finches might be able to imitate a song after five days with male song tutor*

Our results do not directly support that juvenile birds were able to significantly learn from a male tutor they were housed with during a short period of five days. We found that similarity between juveniles' song and tutor and conspecific songs were significantly different at every time point in this study. However, song similarity during adulthood, two months after tutoring, showed a trend towards increased similarity between the subject's song and the tutor's song when compared to before the tutoring period, suggesting birds were able to learn the tutor song, not just general zebra finch song features. This result is supported by previous studies which have shown that zebra finches are able to imitate a tutor song with relatively high fidelity even when exposed to song for a limited period of time later in life, up to sexual maturity around 90 dph, and that similarity to the tutor is high when tutoring occurs later in development (Eales, 1985; Roper and Zann, 2006). We also know that during the sensorimotor period, even more specifically, between

50 and 70 dph, juveniles practice their vocalizations more than any other period and this practice is correlated to similarity to the tutor song at adulthood (Johnson et al. 2002).

One possibility is that these results may be due to the origin of the 'novel' songs. Subjects and tutors are from the same colony, whereas the conspecific songs were taken from a separate colony. It is possible that the tutors used in our colony sing more rudimentary songs that have less variability and more syllable types in common with innate aspects of song. Because juveniles sing a variety of unstructured syllables, the software used is more likely to find similarities between the two songs when the syllables in the tutor song are more basic, such as harmonic stacks (Ölveczky et al., 2005, Tchernichovski, et al. 2001). Thus, it is still likely that subjects in this study were successful in copying aspects of the tutor song even after a short exposure period, supported by a trend in increasing similarity to the tutor song and not to the conspecific song.

***Neural activation of MLd does not change over the course of development***

We observed a tutor song selective response in adulthood, as there was a significant difference in T statistic in right MLd between tutor and conspecific songs as well as a difference in T statistic in response to tutor song in left and right MLd (Figure 14). We, however, did not observe any significant differences in activation between hemispheres or stimuli before or immediately after tutoring, nor any main effects of time. There is one previous longitudinal fMRI study conducted in zebra finches, looking at neural activation to tutor and conspecific song in left and right MLd throughout normal development with normal song tutoring (van der Kant, 2015). Van der Kant (2015) found greater activation to tutor song than to conspecific song in left and right MLd at 60 dph but at no earlier or later time points. They also found that during adulthood, there is a difference between tutor and conspecific song activation only in right MLd, but no

differences between left and right MLd were reported (van der Kant, 2015). We see the same relationship within right MLd during adulthood, but no clear relationships prior to adulthood were found. Possible explanations for this result include that the tutor song selective response does not develop *until* 60 dph, or that its development may be delayed due to our rearing conditions and therefore we cannot observe it within our experimental timeline. Thus, our results not only corroborate this previous longitudinal study but also suggest that this selective response to tutor song is developed in birds that receive tutoring and learn song, not just ones that receive an extended period of tutoring. Additionally, there have been no explicit reports of hemispheric differences in MLd but we find a difference in response to tutor song in left and right MLd adulthood, parallel to hemispheric differences previously found in the NCM using other methods (Bolhuis et al., 2000; 2001; Moorman et al., 2012).

***Activation in the NCM shows bilateral response to tutor song and lateralized response to conspecific song during adulthood***

We found that, in the NCM, response to tutor and conspecific song is bilateral before and immediately after tutoring. During adulthood, there was a difference in % signal change between the left and right NCM in response to conspecific song but a bilateral response to tutor song (Figure 15). Previous fMRI studies in zebra finches have shown no differential response between tutor and conspecific stimuli in the NCM in juveniles, but at adulthood, the NCM is activated by bird's own song and tutor and conspecific songs (van der Kant, 2015; van der Kant et al., 2013). Here, we split tutor and conspecific stimuli and do not look for differential responses alone. Additionally, previous fMRI studies conducted in zebra finches use adults who do not live in isolation and therefore have extensive conspecific exposure. Such studies face a huge confound because they

are unable to discern whether response to conspecific song is due to familiarity or to the same-species nature of the stimulus. In our study, we use conspecific songs that each subject has no experience with and share low similarity to the subject's tutor, thus we are better able to look at conspecific-only effects. Our results suggest that, during adulthood, novel conspecific songs are processed differently in the left and right NCM.

Using IEGs, it has been found that response in the NCM is bilateral upon first exposure to song, but that normally tutored juveniles showed greater, and left lateralized, ZENK activation at ~56 dph to tutor song but not to novel conspecific song (Chirathivat et al., 2015; Gobes et al., 2010; Moorman et al., 2012). Before tutoring, when juveniles hear song for the first time, we see similar bilateral responses to conspecific and tutor stimuli in the NCM. Subjects imaged immediately after tutoring were the same age as in the aforementioned IEG studies, so either we are not able to capture the same neural response because of the fMRI method or the previously observed left lateralization is due to a difference in rearing conditions. Juveniles in previous studies had more song experience at this age than our subjects who may not have developed this lateralized response yet. However, at adulthood, there is higher neuronal activation to tutor song in the NCM compared to silence, but it is not lateralized (Moorman et al., 2012). We see a similar bilateral response in this study in response to tutor song at adulthood, supporting previous findings.

***Neural activity in the NCM is related to tutor song similarity in adulthood***

In this study, significant positive correlations in the left and right NCM between tutor song similarity and neural activation in response to tutor song were found (Figure 17). In both the left and right NCM, the stronger the activation to tutor song, the better a bird's song matched its tutor's.

Previous fMRI literature shows that there is no significant correlation between tutor song similarity and tutor/conspecific differential activation in the left NCM (van der Kant et al., 2013). IEG experiments, however, show that in the NCM, ZENK activity in response only to tutor song, but not conspecific song, is positively correlated with similarity to the tutor song in adult zebra finches (Bolhuis et al., 2000, 2001; Terpstra et al., 2004). A later study found that there was a positive relationship between the degree of left lateralized ZENK activity in the NCM and the similarity to a tutor song (Moorman et al., 2012). Our results support previous findings showing almost parallel positive correlations in adults in response to tutor song in left and right NCM, and no significant relationships to conspecific song exposure. It is not possible to directly compare this result from this study to previous IEG studies because they either calculate neuronal activity for both hemispheres at the same time or use lateralization ratios and do not look at hemispheres separately. The observed difference in results between this study and that of Moorman et al. (2012) could be a product of the method: IEG studies show changes in protein product in individual interneurons, whereas fMRI obtains data from neuronal populations. The relationship between learning and neuronal activation depends on the degree to which individual cells respond to tutor song more in the left NCM than in the right NCM is separate from the relationship between learning and the size or strength of neuronal networks activated in both the left and right NCM. Thus, the activation of both the individual neurons and neuronal populations are both related to learning strength.

***Neuronal activation before tutoring predicts learning outcome at adulthood***

We found significant correlations between both T statistic and % signal change in left MLd in response to tutor song before tutoring and tutor song similarity during adulthood (Figure 16). Similar correlations were not significant in response to conspecific song; however, they were

trending towards significance. One previous study showed, using electrophysiology, that song-selective neurons in HVC of untutored juvenile zebra finches show preference for conspecific adult song (Adret et al., 2012). This suggests that there could be innate preferences for nonrelated adult song before any song experience in other regions as well. It is possible a heightened activation in auditory nuclei, including MLd, to any song when a juvenile hears song for the first time could predict learning outcome later in life.

We originally hypothesized that we could use neural activation to tutor song immediately after tutoring as a predictor of similarity to tutor song in adulthood but found no significant correlations between activation in any region and similarity to the tutor song during adulthood. One possibility is that response to tutor song after tutoring, the activation of a tutor song memory, is simply not predictive of learning outcome, it is only the initial activation to a song that is predictive. An alternate and more likely explanation is that there is no differential response to tutor song immediately after tutoring. As previously mentioned, other studies have not seen a tutor song specific response until several days after tutoring and in juveniles with much more tutor exposure than provided in this study (Gobes et al., 2010; van der Kant, 2015). For the subjects in this study, the tutor song selective response may not develop until later. In that case, future studies can observe neural responses at time points between two days after tutoring and adulthood to see whether late tutoring leads to development of the tutor-song specific response later than in normally tutored zebra finches.

***Future directions***

We must remain critical of the resolution in this dataset in several dimensions. Due to the crude design of ROIs, which were all modeled as spheres, there was no way to determine

coregistration among all regions and all imaging sessions. This presents several problems. First, these regions are not physically spherical in reality and modeling them as such might exclude parts of a region and include parts of other regions. Due to the anatomical proximity of other nuclei, there is no certainty that the ROIs created did not include any part of any other nuclei. For example, the NCM shares an anatomical border with Field L, which would impact the results presented here because Field L is a primary auditory nucleus that responds to all song stimulation. Second, because each ROI was built separately for each imaging session and because of geometric variation between imaging sessions and subjects, there is no way to be consistent. Without the additional check of anatomical coregistration between and within subjects, there is no certainty that we are observing the same physical brain area in every subject across every time point. Furthermore, because ROIs could only be modeled as spheres, we were not able to create and observe activity in a control nucleus, namely Field L, which cannot be modeled as a sphere. Without such a control, we cannot be sure that results we see are not due to chance. Finally, due to time constraints, fMRI data sets only contained four trials of either stimulus, which may not be temporally resolved enough. While previous literature does not show a minimum requirement number of trials to ascertain significant results, previous studies use at least 25, if not more, trial stimulus presentations (van der Kant et al., 2013; Poirier et al., 2009).

Due to the longitudinal nature of our study, to conduct statistics all measures must be accounted for each subject and at each time point. In the present study, at each level of analysis, technical difficulties were encountered, resulting in inability to gain all measures for all birds at each time point. This ultimately results in a final sample size that is potentially too small to show many trends and correlations that may underlie behavioral outcomes, or alternately, gives many false positives. To account for the small longitudinal sample size, we also looked at stimulus and

hemisphere differences within each time point, but the data then becomes cross sectional and not longitudinal. Future objectives of the project should include determining where technical errors occur and eliminating them to have more complete sets of data and thus, more robust results.

Finally, finer analysis methods should be implemented because they could reveal more robust results. In this study, we did not look at differential response between tutor and conspecific song, but rather looked at responses to each, separately, which makes it hard to compare the present findings to those in previous studies. Exploring differential response analysis may further elucidate relationships between neural activations and learning strength. Moreover, while T statistic and % signal change are commonly used in human and zebra finch fMRI research, there are other measures of neural activity that can be taken from this data set using finer methods of analysis, including amplitude (magnitude) and volume of BOLD activation in each region of interest. Early fMRI studies in zebra finches used these measures to demonstrate differences in auditory perception between different stimuli and even between normal zebra finches and those that stutter or repeat syllables (Poirier et al., 2009; Voss et al., 2010). Observing contrast and signal changes to uncover differences in auditory response may not be enough and other properties of the BOLD response should be considered to get a more complete picture of how populations of neurons in these auditory regions are responding to stimuli.

Future goals of this project include not only incorporating new measures but also improving the methods by implementing more stimuli trials, determining more anatomically-relevant and accurate ROIs that are systemically spatially consistent, and eliminating technical errors that result in missing measures for subjects. Such improvements can provide a clearer picture of how populations of neurons in the zebra finch are responding differently through the vocal learning process, which will allow us to better understand how model vocalizations develop.

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# Appendix #1: fMR Imaging Protocol

Stringing speaker (If setting up from bare):

1. Wrap stereo plug end around ladder hinge or on the hook on the tank so it will not go into the magnet (this is important because it is slightly magnetized)
2. Wrap connector in removable tape carefully so it doesn't stick or get stuck going down the bore. Lower the connecter of the stereo plug end down the bore, until it reaches the bottom. Pull out of bottom and connect to the bed end side of the connector.
3. Make sure the connection is tight. Can use medical tape around it if you're worried about it sticking to the walls of the bore.
4. Can test speaker by plugging in cell phone (at least 1 meter from magnet) and playing sound while holding the small magnet next to the speaker. You should do this every time before imaging to make sure sound is working.

Setting up probe:

1. Check the isoflurane level in the vaporizer and charcoal filter time (max 12 hr, gain of 50g) before beginning any experiment.
a. Record the date on the filter
b. Check anesthesia set up and connections
c. Lines to chamber are color-coded with tape; make sure they are attached properly. Exhaust line should always remain attached to the bed. The in gas line should be connected to bed or to the isoflurane chamber using the higher hole and the adapter/connector.
d. Turn up magnet to 33.1C using temp controller by back wall, this will translate to 36C inside the bore.

2. Charge induction chamber with oxygen.
- Open oxygen with top valve. Open main valve completely. Don’t let O2 level get below 4,000.
- Set flow rate to 0.4

3. Once bird has been collected and filter and gas hose has been connected, start anesthesia by adding isoflurane. Release dial by pressing white lever on silver isoflurane cannister; turn lever to between 2-3%. The anesthesia usually takes effect within ten minutes, but breathing should be monitored visually to determine if bird is under. While waiting for bird to fall asleep, set up the breathing monitor.

4. To monitor respiration, the BioTrig BT1 system (BrukerBioSpin MRI GmbH, Germany) is used. Attach pressure pad line to RESP input on Acquisition Module (grey box next to the probe)

5. On the Dell Latitude laptop (there is no password for it, just click enter), open BioTrig Build 1.01 by clicking Shortcut to BioTrig on the desktop.
6. In the Options/Tools pull-down menu, select Digital Rate ChB to display breaths/min.
7. Under the Channels menu.
a. Open Channel Setup and in the Resp column click on the slider to make the Scaling Factor 4
2. Set the Gain Factor to 2 so you can see individual breaths. Click Apply and Exit.
*****NOTE: When imaging juveniles, amplitude of breathing may appear low and BioTrig may say 0 bpm, if so, change scaling factor to 6 and Gain to 4. You should see the amplitude augment and the bpm should rise above 0.**
**You can test by varying pressuring with your thumb on the transducer and watching the pattern is repeated on the screen.

8. Once the bird is unconscious (not shivering, tail is still) and respiration is slowed, switch anesthesia lines to nose cone.

9. Position pressure pad on the bed so that it is centered on the bird's chest (should be set up so that it is always underneath the chest of any bird).

10. Quickly remove bird from induction chamber and place prone into the bed.
11. Place the bird's beak in the beak hole (can use mirror to make sure beak placement is good- if it's not the bird may not only be crooked but may also wake up in the magnet).

12. If the bird truly awakens while securing it to the probe place it back into the isoflurane induction chamber until the bird is in a comfortable sleep.
*Can also cover bird with hand while his head is in the head cone so he's breathing isoflurane and settles down.

13. Secure bird in bed, make sure the feet are not tangled in tubing. Use cut up kimwipes to cover the bird's feathers and then use medical tape to secure. Do not be shy about taping them in well, prevents movement during imaging and ensures the bird will fit into the probe. Generally, use one ~5cm piece of medical tape across the largest part of the bird (shoulders/chest). Tape tightly enough to secure the bird and immobilize but not too tight so he can't breathe.

14. Carefully, slide the bird bed into the probe. Check the breathing rate and make sure it isn't below 45 breaths/minute. (Rate seems to drop when probe is inserted vertically into magnet.) Remember to wait a while after adjusting isofluorane level; there will be a delay before you see an effect in breathing after changing it. Make sure breathing is good and stable before inserting probe into the magnet.

After putting probe into the magnet:

1. Tune and Match water peak (Tools -> Acq -> Wobble)
2. Make sure your patient and study have been set up.
3. Run Tripilot Image (RARE-tripilot under BB_PVM)
4. Use Tripilot Image to adjust geometry settings for future scans
5. Check Wobble
6. Run RARE_8_bas (in Songbird folder) image to obtain anatomical images.
7. fMRI protocols are located in Songbird folder- current one to use is **fMRI Trigger as of Spring 2015.4**

To clone scans:
Two ways:
1A. set up scan as you want.
2A. right click on the scan and go to clone scan, click

1B. Go to Macro Manager (the Top Hat Magic looking thing in the Scan Control)
2B. click the + sign on lobster
3B. go to the 'clone', hit the 'edit button', NOT THE PLAY BUTTON
4B. Type number of scans you want (not total, but how many MORE scans)
5B. hit the save button.
5C. click on the scan you want cloned in the Scan Details window, then press the play button in the Macro Manager.

Queued scanning:
TWO WAYS:
MUST BE LOGGED ONTO LOBSTER ACCOUNT

1A. In Paravision, go to the magic hat icon on the Paravision Control. This will pull up the macro manager, click the + on lobster
2A. Click on Queued Scan Manager and then hit the play button.
3A. Click on Select Patient and then queue scans as needed. (The scans must be in scan manager and all set up before queuing them)
4A. Press Run acquisition. This starts the acquisition series. Do this after you have set Presentation to run.
** This method conducts setup between each individual scan. In Presentation code, must change the pulse number to 1 from 2. Stimulus begins several seconds before image acquisition begins.

1B. Go to the Macro manager. Click on the + on the lobster
2B. go to 'Scan Series' macro, click on the edit button

3B. Type number of scans you want to queue and sequentially scan.
4B. If you want a delay between scans, type number of ms into the macro
5B. In the Scan Control panel, click on the first scan in the series
6B. then press the play button. In the scan control, it will display in red how many scans are in the series
**This method does not conduct setup between each scan; in Presentation code, must account for this by changing the number of pulses per scan to 1. Stimulus begins less than one second before image acquisition starts.

NBS Presentation:
1. On the lab laptop, go to Presentation.
2. Make sure Arduino is properly connected to the green wire and the computer and is set up correctly in Presentation as the fMRI trigger. If setting up with Stela_fMRI 2015 experiment, settings for the port are already done, if not, you have to set it up again in port settings, see the instructions in Stela's lab notebook (which will eventually be made into a doc accessible on that computer). No response buttons should be active.
3. Load the stimuli folder and the code from the folder on the desktop. (Already done if using the Stela experiment.)
4. In the editor, make sure you are not running fMRI simulation and read over the code to make sure if it fits the experiment. Make sure stimuli are loaded correctly.
5. Press Run (green arrow) BEFORE starting queued acquisition.

# Appendix #2: Code for Arduino and Experimental Paradigm in NBS

## Code for Arduino:

Further instructions found at:
http://www.neurobs.com/wiki/Presentation/Hardware/Other_Devices/Arduino#Arduino_program

```
onst int PIN = 2;
int state = 0;

void setup()
{
 Serial.begin(115200);

 pinMode(PIN, INPUT);
 state = bitRead( PIND , PIN );
}

void loop()
{
 int new_state = bitRead( PIND , PIN );
 if (new_state != state)
 {
  state = new_state;
  if (state)
  {
   Serial.write( 1 );
  }
  else
  {
   Serial.write( 0 );
  }
 }
}
```

# Code for Experimental Paradigm:

```
#Paradigm with dummy trials
#24 images, 4 blocks of song
#Stela Petkova

scenario = "fmri";
scenario_type = fMRI; #_emulation;
#fmri_emulation allows you to try out the code without the trigger/magnet
#you need to designate a scan period for emulation
#scan_period = 4;
pulses_per_scan = 2;
#pulses per scan is 2 if doing Queue GUI, 1 if doing George's way
pulse_code = 1;
#no_logfile = true;
begin;


#put file name in quote in parentheses,
wavefile { filename = "WCGreen93_1.25x_15sec_SP.wav"; } Song;
wavefile { filename = "Silence_16sec.wav";} Silence;


#letters that'll be displayed
#write what you want to be displayed in 'caption'
array {

   text { caption = "Dummy"; description = "A"; font_size = 50;} A;
  } letters;

array {
   text { caption = "Song"; description = "B"; font_size = 50; } B;
  } letters2;

array {
   text { caption = "Silence"; description = "C"; font_size = 50; } C;
  } letters3;

#number of MR pulse is number of scan in the paradigm

#Block 1- silence
trial {
   sound { wavefile Silence; } sound1;
           time = 0;
           mri_pulse = 1;
} trial1;

trial {
   sound { wavefile Silence; } sound2;
           time = 0;
```

```
            mri_pulse = 2;
} trial2;

#block 2-song
trial {
  sound { wavefile Song; } sound3;
            time = 0;
            mri_pulse = 3;

            stimulus_event {
     picture {
       text B;
       x = 0; y = 0;
     } pic3274;
     time = 0;
     duration = 10000;
  } event3241;
} trial3;


trial {
  sound { wavefile Song; } sound4;
            time = 0;
            mri_pulse = 4;
} trial4;

#block 3: Silence- dummy
trial {
  #trial_duration = 20000;

  stimulus_event {
     picture {
       text A;
       x = 0; y = 0;
     } pic;
     time = 0;
     duration = 10000;
                        mri_pulse = 5;
  } event1;
} trial5;

trial {
 # trial_duration = 10000;

  stimulus_event {
     picture {
       text A;
       x = 0; y = 0;
     } pic1;
     time = 0;
     duration = 10000;
```

```
                    mri_pulse = 6;
  } event2;
} trial6;

#Block 4- Silence
trial {
  sound { wavefile Silence; } sound7;
          time = 0;
          mri_pulse = 7;
} trial7;

trial {
  sound { wavefile Silence; } sound8;
          time = 0;
          mri_pulse = 8;
} trial8;

#Block 5- Song
trial {
          sound { wavefile Song; } sound9;
          time = 0;
          mri_pulse = 9;

          stimulus_event {
    picture {
      text B;
      x = 0; y = 0;
    } pic324;
    time = 0;
    duration = 10000;
  } event321;

} trial9;


trial {
          sound { wavefile Song; } sound10;
          time = 0;
          mri_pulse = 10;

          stimulus_event {
    picture {
      text B;
      x = 0; y = 0;
    } pic374;
    time = 0;
    duration = 10000;
  } event341;
} trial10;

#Block6- No sound
```

```
trial {
  #trial_duration = 10000;

  stimulus_event {
    picture {
      text A;
      x = 0; y = 0;
    } pic3;
    time = 0;
    duration = 10000;
                mri_pulse = 11;
  } event3;
} trial11;

trial {

  #trial_duration = 10000;

  stimulus_event {
    picture {
      text A;
      x = 0; y = 0;
    } pic4;
    time = 0;
    duration = 10000;
                mri_pulse = 12;
  } event4;
} trial12;

#Block 7- silence
trial {
  sound { wavefile Silence; } sound13;
        time = 0;
        mri_pulse = 13;
} trial13;

trial {
  sound { wavefile Silence; } sound14;
        time = 0;
        mri_pulse = 14;
} trial14;

#Block 8- Song

trial {
  sound { wavefile Song; } sound15;
        time = 0;
        mri_pulse = 15;
        stimulus_event {
    picture {
      text B;
```

```
      x = 0; y = 0;
    } pic327;
    time = 0;
    duration = 10000;
  } event324;
} trial15;

trial {
  sound { wavefile Song; } sound16;
          time = 0;
          mri_pulse = 16;

          stimulus_event {
    picture {
      text B;
      x = 0; y = 0;
    } pic274;
    time = 0;
    duration = 10000;
  } event241;
} trial16;

#Block 9- No sound

trial {

  #trial_duration = 10000;

  stimulus_event {
    picture {
      text A;
      x = 0; y = 0;
    } pic5;
    time = 0;
    duration = 10000;
                    mri_pulse = 17;
  } event5;
} trial17;

trial {

  #trial_duration = 2000;

  stimulus_event {
    picture {
      text A;
      x = 0; y = 0;
    } pic6;
    time = 0;
    duration = 1000;
                    mri_pulse = 18;
```

```
  } event6;
} trial18;

#Block 10- Silence
trial {
  sound { wavefile Silence; } sound19;
        time = 0;
        mri_pulse = 19;
} trial19;

trial {
  sound { wavefile Silence; } sound20;
        time = 0;
        mri_pulse = 20;
} trial20;

#Block 11- Song
trial {
  sound { wavefile Song; } sound21;
        time = 0;
        mri_pulse = 21;
} trial21;

trial {
  sound { wavefile Song; } sound22;
        time = 0;
        mri_pulse = 22;
} trial22;

#Block 12- NO sound

trial {

  #trial_duration = 10000;

  stimulus_event {
    picture {
      text A;
      x = 0; y = 0;
    } pic7;
    time = 0;
    duration = 10000;
                mri_pulse = 24;
  } event7;
} trial23;

trial {
  #trial_duration = 10000;

  stimulus_event {
    picture {
```

```
        text A;
        x = 0; y = 0;
      } pic8;
      time = 0;
      duration = 10000;
                    mri_pulse = 24;
   } event12;
} trial24;

begin_pcl;

sub
   play( string message )
begin
   display_window.erase();
   display_window.draw_text( message );
   trial1.present();
            trial2.present();
            trial3.present();
            trial4.present();
            trial5.present();
            trial6.present();
            trial7.present();
            trial8.present();
            trial9.present();
            trial10.present();
            trial11.present();
            trial12.present();
            trial13.present();
            trial14.present();
            trial15.present();
            trial16.present();
            trial17.present();
            trial18.present();
            trial19.present();
            trial20.present();
            trial21.present();
            trial22.present();
            trial23.present();
            trial24.present();
end;
```

# Appendix #3: Stimuli Creation Protocol

Song stimuli were created using PRAAT software.

1. After finding the song recordings for the bird you want to make a stimulus for, open PRAAT.
2. Load song files.
3. Select one full motif from each song recording file ('View and edit" button) and copy it.
4. Paste it into what will become your final stimulus. (either 15 or 30 seconds)
5. Be sure to have enough natural silence between each motif.
6. Filter using the 'Stop Hann Band'. Use parameters 0 400 100
7. Go to Annotate--> Text grid
8. Click "Text Grid and sound" and then "View and Edit with Sound"
9. Annotate the song accordingly.
10. Open the PRAAT script on Academic Store (Under Praat scripten) 'equalize rms scripten' Run with both the Text Grid and the sound file selected.
11. Amplify the stimulus to 1.25x by going to Modify --> Multiple
12. Save as a .wav file (by putting .wav at the end of the file name)

Name should include the bird name, amplification, length of stimulus and your initials.

# Appendix #4: Changing Voxel Size Protocol

Changes to voxel size were conducted in MATLAB using the opensource Nifti Toolbox.

*anat can be any variable, I defined each different anatomical some other variable

1. define the image anat= load_nii ('filename.nii',1,1,1,1,1,1)
    a. Each 1 represents a different property. More information about each property can be found on the manual and documentation for NiftiToolbox.
1. in the Workspace, click on 'anat' variable
1. then double click **hdr**
2. then double click **dime**
3. then double click **pixdim**
4. Change the dimensions in columns 2, 3 and 4 by multiplying by 10 (for anatomicals should be 0.977 x 0.977 x 7.5mm and for functional it should be 3.9 x 3.9 7.5 mm and yes, even if it is not exactly that number, which chances are it won't for every image)
5. close out of workspace variables
6. in the command line, type save_nii (anat, 'new file name.nii')
7. In the Current folder on the left, the new file should appear!

To check voxel size, in SPM, click on Display and open the new file. Voxel size is listed on the right box in mm.

Each image should be its own variable name.

**Batching:**

(Currently only setup for the 24 images, but can be amended easily for more)

There is a Word document in Stela's folder on Academic Store that you can copy and paste the first part of into the MATLAB command window to load all of the files in one paradigm and it'll batch load them.

Open each hdr and pixdim and change the voxel sizes.

Close all the variable windows

Then when you finish all of them, you copy and paste the second part of the Word document with save_nii functions.

You should change the names of the images to fit this: select all of them, right click 'rename' and rename as f. they will all rename with f (n). nii to work for this method

# Appendix #5: Global Brain fMRI Analysis

In this thesis, Steps 3-5 were skipped. Additionally, global group analysis was not conducted.

1. **Realign (Est & Res)**
    1. New session
        1. Specify files – select image files in the paradigm folder
        2. Run (green arrow)
    2. **Coregister (Estimate)**
        1. Reference image – mean fMRI scan (mean image file)
        2. Source image – anatomical that corresponds to the paradigm set
        3. Run (green arrow)
    3. **Segment**
        1. Data – **anatomical**
        2. Gaussians – [2 2 2 4]
        3. Affine Regularization – **No Affine Registration**
        4. Run
    4. **Normalize (Write)**
        1. Data – new "subject"
            1. Parameter file – image_sec_sn.mat
            2. Images to write – realigned functional images: rimage.img
            3. Writing Options
        2. Voxel Sizes – change from [2 2 2] to:
            1. **[0.4 0.4 0.75] for 16 s FOV 2.50cm**
            2. **[ 4 4 7.5] for upscaled images**
            3. Run
    5. **Smooth (skip)**
        1. Images to smooth – spatially normalized images – wrimage.*
        2. FWHM – change to twice voxel size
            1. [1.6 1.6 1.6]
        3. Run
    6. **Specify 1st level**
        1. Timing Parameters
            1. Units for design – scan
            2. Interscan interval – 2
        2. Data & Design – New subject/session
        3. (can also do OPEN: Academicstore > Data > Computer 4 > Stela > DATA AND ANALYSIS: SONG VS SIL 2015 file (only for 24 images with 4 song blocks)
            1. Scans – normalized images – wr images
            2. Conditions (alternatively, save a template file for conditions)
                1. New condition
                    1. Name – song
                    2. Onsets – paradigm dependent
                    3. Durations – 2
                2. New condition (this condition is technically not required in a two stimulus design, it overspecifies the model when it doesn't really need to)
                    1. Name – silence
                    2. Onsets – paradigm dependent
                    3. Durations – 2

3. High Pass Filter : 352
4. Directory – select original paradigm folder (using the ./ option in the right side of the dialog box)
5. Run

7. **Estimate**
    1. Select SPM.mat – select SPM.mat file created in previous step
    2. Run
8. **Results**
    1. Select SPM.mat file created in original folder
    2. Invokes contrast manager:
        1. Define new contrast (t-contrast)
            1. 1 (song > sil) '1 0' in big box
            2. 2 (song - sil) '1 -1' in big box
            3. **ORDER DOES MATTER**, if you do them in reverse, make a note of that somewhere in lab notebook and in the digital files)
            4. press done
        2. Select which contrast you want to look at twice
        3. Mask – no
        4. Title? – arbitrary just use title they fill in
        5. FWE – no
            1. if you say yes, this does multiple comparisons and you basically don't get any results!
        6. P-value - usually 0.05 but flexible
        7. Extent Threshold – usually 5 (but flexible)
        8. Overlay – sections - anatomical image
        9.
9. **Group Analysis NEW Nov 2015!**
    1. 'Specify 2nd level'
    2. you need to make a new folder in the imaging session folders with the title of the comparison you're going to make like 'all tutor' or 'all tutor greater all con' etc
    3. Directory -- that new folder
    4. Design --
        1. one sample t test: comparing multiple of the same stimulus to zero (so like taking the average and seeing if it's significantly nonzero)
        2. two sample t test: comparing one type of stimulus with multiple subjects to another stimulus
        3. Choose the appropriate test
        4. Always write down what your group 1 and group 2 are in a lab notebook!
        5. In this model, you're not inputting in images, but the CONTRASTS from results page.
            1. This is why keeping contrasts the same between paradigms, sessions and birds is important and why careful notetaking is important!

# Appendix #6: ROI Center Coordinates Used

Coordinates are given as x (space) y (space) z (space) in mm from SPM defined origin.

Diameter for MLd ROIs was 5mm, diameter for NCM ROIs was 6 mm.

| Bird + Age | Left MLD | Right MLD | Left NCM | Right NCM |
|---|---|---|---|---|
| WCOrange29 pretutor | -30.9 8.3 18.6 | 31.9 17.0 24.1 | -12.8 63.2 4.5 | 13.8 60.1 6.9 |
| WCOrange29 adult | -23.8 28.7 23.9 | 25.6 -4.2 23.9 | 14.6 56.2 3.5 | 32.8 47.5 3.5 |
| WCOrange31 pretutor | -8.9 33.4 43.3 | 19.3 -16.0 24.4 | 35.0 42.8 21.3 | 44.4 27.9 15.8 |
| WCOrange31 posttutor | -23.8 23.2 22.4 | 26.4 -4.2 22.4 | 16.2 54.6 2.0 | 33.4 44.4 2.0 |
| WCOrange31 adult | -27.0 20.1 18.0 | 27.9 7.5 21.2 | 3.6 57.7 -6.3 | 26.4 57.7 5.5 |
| WCOrange50 pretutor | -30.9 6.8 17.5 | 22.4 24.0 31.6 | -20.7 52.2 5.0 | 1.3 55.4 9.7 |
| WCOrange50 posttutor | -27.7 11.5 20.2 | 31.1 17.7 34.3 | -13.6 54.6 6.0 | 9.9 56.5 9.9 |
| WCOrange54 pretutor | -26.2 18.5 25.1 | 30.3 2.1 10.2 | -2.6 60.9 -3.2 | 23.2 52.2 -5.5 |
| WCOrange54 adult | -34.0 8.3 23.3 | 29.5 0.5 24.8 | -8.9 51.5 4.4 | 15.4 50.7 4.4 |
| WCOrange69 posttutor | -29.8 11.6 68.8 | 27.4 14.7 68.8 | -18.1 57.8 51.5 | 12.5 59.4 51.5 |
| WCOrange75 pretutor | -36.4 8.3 9.4 | 28.7 10.7 9.4 | -19.9 53.0 -7.8 | 13.0 53.8 -7.8 |
| WCOrange75 posttutor | -33.6 4.9 25.7 | 26.0 17.4 36.7 | -19.5 50.3 5.3 | 11.1 62.1 21.0 |
| WCOrange75 adult | -26.1 8.0 29.3 | 37.5 13.5 29.3 | -10.4 55.8 12.1 | 20.2 56.6 12.1 |
| WCOrange77 pretutor | -32.4 2.1 7.4 | 32.6 10.7 6.9 | -18.3 49.1 -9.6 | 9.9 50.7 -9.6 |
| WCOrange77 posttutor | -33.2 1.3 15.4 | 34.2 7.5 15.4 | -18.3 46.0 -5.0 | 10.7 49.9 -5.0 |
| WCOrange77 adult | -29.5 4.7 19.9 | 38.7 7.8 25.4 | -9.9 48.6 -2.1 | 19.9 49.4 2.7 |
| WCBlack08 pretutor | -32.6 10.4 27.6 | 32.5 11.2 27.6 | -9.9 52.0 13.4 | 15.2 52.0 13.4 |
| WCBlack08 postutor | -24.2 19.3 1.4 | 36.2 5.2 -5.6 | -1.4 61.6 -20.6 | 25.2 56.9 -21.3 |
| WCBlack08 adult | -30.9 20.1 27.7 | 25.6 16.2 27.7 | -16.8 60.1 14.4 | 16.2 59.3 14.4 |
| WCBlack13 pretutor | -31.7 2.8 15.4 | 25.6 17.0 30.3 | -19.1 51.5 1.3 | 8.3 55.4 6.8 |
| WCBlack13 posttutor | -36.9 8.8 16.7 | 28.1 9.6 28.5 | -15.7 54.3 0.6 | 10.9 54.3 2.6 |
| WCBlack13 adult | -35.7 23.2 | 23.1 16.2 8.5 | -12.9 63.2 -4.8 | 15.3 60.1 -4.6 |
| WCBlack14 pretutor | -20.4 15.2 36.9 | 34.4 11.3 36.9 | -8.7 56.8 11.0 | 24.3 56.0 11.0 |
| WCBlack14 posttutor | -29.4 12.9 2.4 | 25.5 2.7 -18.0 | -4.3 56.0 -18.0 | 21.6 49.8 -18.0 |
| WCBlack25 pretutor | -46.7 6.8 -6.5 | 13.6 2.1 -5.7 | -25.6 48.4 -15.9 | -0.5 49.2 -15.9 |
| WCBlack25 posttutor | 1.0 28.1 6.8 | 55.9 19.4 6.8 | 17.5 61.8 -13.6 | 53.6 61.0 -13.6 |
| WCBlack25 adullt | -30.1 17.7 30.2 | 24.0 11.5 30.2 | -15.2 58.5 15.3 | 11.5 60.9 17.6 |
| WCBlack26 adult | -35.0 13.0 37.9 | 20.7 6.8 35.6 | -13.8 55.4 23.8 | 7.4 53.0 20.6 |
| WCBlack36 pretutor | -23.6 -13.3 17.9 | 37.5 -1.6 15.6 | -14.2 35.3 -11.4 | 14.6 39.2 -11.9 |
| WCBlack36 posttutor | -40.9 7.3 0.7 | 18.7 0.3 0.7 | -18.2 52.8 -20.5 | 4.6 52.0 -24.5 |
| WCBlack36 adult | -35.0 13.8 44.3 | 26.9 9.9 44.3 | -9.9 61.6 24.7 | 14.4 58.5 24.7 |
| WCBlack42 adult | -34.8 9.9 25.6 | 25.8 9.9 25.6 | -17.5 56.1 4.4 | 9.9 56.1 4.4 |
| WCBlack43 pretutor | -28.4 13.2 38.2 | 28.4 13.2 38.2 | 10.4 57.1 21.7 | 15.5 54.7 21.7 |
| WCBlack43 posttutor | -35.7 23.2 8.5 | 23.1 16.2 8.5 | -12.9 63.2 -4.8 | 15.3 60.1 -4.6 |
| WCBlack43 adult | -25.8 13.8 47.0 | 30.7 14.6 47.0 | -7.0 56.9 32.1 | 19.7 56.9 32.1 |

Highlighted rows signify data added after the original submission of the thesis.

# Appendix #7: fMRI Region of Interest Analysis Protocol

**To build ROI in marsbar:**

1. **ROI definition from coordinates**
    a. Build
    b. Type : Sphere
    c. type in coordinates of center of region
    d. diameter is diameter (in MM not in voxels)
    e. Save and done

2. **ROI definition from SPM cluster:**
    a. pull up the contrast you want through SPM
    b. ROI definition in marsbar:
    c. Get SPM cluster
    d. in the Analysis window of SPM, there is a new button 'write ROI' in the top bar
        i. has issue where it just says 'no activated voxel at this location if you write one cluster
        ii. if you select write all clusters, it saves each cluster as a separate ROI file that is by rough coordinates
    e. Build by cluster or by coordinates from there
    f. THIS WORKS ONLY IF THERE IS A CLUSTER ACTIVATION IN THAT REGION

3. T**o combine ROIs**
    a. ROI definition
    b. Transform
    c. input ROI files to combine
    d. function :
        i. ' r1 & r2' to look at the overlapping activation of ROIs
        ii. ' r1 | r2 to look activation in ROI1 but not ROI2
        iii. (r1 % r2) ~r3 looksat all voxels in both ROI1 and ROI2 but not in ROI3.

**4. Running ROI analysis**
    a. Build ROIs to analyze
    b. Design
        i. set design from file
        ii. choose SPM file for that imaging session/paradigm
    c. Data
        i. Extract
        ii. Full options
        iii. (can select multiple ROIs) this doesn't combine the ROIs into one ROI, but rather gives you a table at the end
        iv. images → images from SPM design
        v. RAW data (Scaling is 0)
        vi. should get a table in the graphics with a summary of what you just input
    d. Results
        i. estimate
        ii. save results to file
    e. getting the results table from the MATLAB command line

i. load (insert what you just named the results file)
ii. SPM
iii. SPM.mars.Y
iv. SPM.mars.Y.Y

f. getting results from Marsbar
i. Results
ii. Statistic table → input contrasts
iii. Statistics table will appear in the MATLAB command window including contrast values, T statistics and p values.

**5. Other features:**

a. % signal change:
i. after you save the results and load it, you can go to results % signal change (shows up in the MATLAB command window)

b. plot residuals:
. helps you plot things (you can get a signal intensity by time graph)
i. this can be helpful in seeing response change to song stimuli over the time course of the experiment

c. SPM graph:
. give you a graph of the responses at a cluster or ROI and you can define by event or by time, fitted, predicted, etc. not sure if that's going to be helpful

# Appendix #8: Supplemental Figures and Results

***Behavioral Analysis:***

After the initial submission of the thesis, the behavioral data of five adult birds was added. We found a significant main effect of stimulus exposure (repeated measures ANOVA: $F_{(1,12)} = 15.530$, $p = .002$, $n = 12$). With a more complete data set, % similarity remained significantly different between tutor and conspecific song at every time point (Figure 19, Before tutoring: $p < .05$, After tutoring: $p = .01$, Adulthood: $p < .0001$). Additionally, we found a significant difference between tutor similarity at 48dph and 120dph, suggesting that juvenile birds did learn from their tutor by adulthood ($n = 13$, $p < .05$).

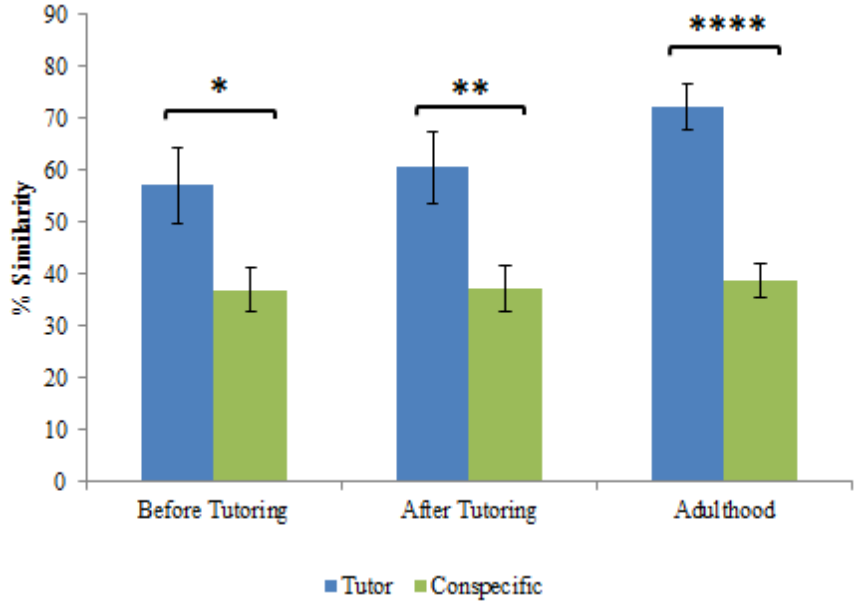


**Figure 19: Updated similarity between subjects and tutor and conspecific songs over time.** Similarity of subject song is significantly different between tutor and conspecific songs at all three time points. Mean ± SEM shown. Data for all birds shown (before tutoring: n = 12, after tutoring: n = 12, adulthood: n = 13).

* signifies $p < .05$
** signifies $p < .01$
**** signifies $p < .0001$

***fMR Imaging Results:***

With the addition of the data from the remaining birds at adulthood, previous results, for the most part, were found to be nonsignificant. However, one significant difference found remained significant; the % signal change between left and right NCM in response to conspecific song at adulthood was still different (paired t test: left: -1.21 ± 2.27 SEM right: -6.64 ± 2.12 SEM, $p = .009$, $n = 12$).

Additionally, the correlations found in this thesis were no longer significant with the inclusion of the five additional adults.